Distribution of *Mycobacterium* tuberculosis lineages overview in the North of Vietnam

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ABBREVIATIONS

AFB	Acid-fast bacillus
BCG	Bacille Calmette-Guérin
CAS	Central-Asia
CXR	Chest X-ray
DOTS	Directly Observed Treatment, Short-course
DR	Direct Repeat
DST	Drug Susceptibility Test
EAI	East African Indian
ECL	Enhanced chemiluminescence
EPI	Expanded Program on Immunization
HNTRH	Hanoi Tuberculosis and Respiratory Diseases Hospital
IDU	Intravenous Drug User
IS*6110*	Insertion Sequence *6110*
IUATLD	International Union Against Tuberculosis and Lung Diseases
LAM	Latin-American-Mediterranean
LJ	Löwenstein-Jensen
MDR	Multi-drug-resistant
MTB	Mycobacterium tuberculosis
NACL-NAOH	N-ACETYL-L-CYSTEIN-SODIUM HYDROXIDE
NIHE	Institute of Hygiene and Epidemiology
NIPH	Norwegian Institute of Public Health
NTP	National Tuberculosis Program
NTRH	National Tuberculosis and Respiratory Diseases Hospital
OPV	Oral Polio Vaccine
PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
SES	Socio-Economic-Status
SPSS	Statistical Package for the Social Sciences
TB	Tuberculosis
TBTRH	Thai Binh Tuberculosis and Respiratory Diseases Hospital
TST	Tuberculin Skin Test
WHO	World Health Organization

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ABSTRACT

Distribution of *Mycobacterium* tuberculosis lineages, Overview in Northern - Vietnam

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Vietnam was among the high-burden tuberculosis countries. Molecular typing is not available on a routine basis in the Vietnamese National Tuberculosis Program's laboratories. DNA fingerprinting of *Mycobacterium tuberculosis* is an international standard epidemiologic tool, which exploits variability in both the number and genomic position of Insertion Sequence *6110*. This method allows generating strain specific DNA patterns (DNA fingerprints). Besides, IS*6110*-Restriction Fragment Length Polymorphism (RFLP) analysis has proven useful for the recognition of outbreaks in communities, modes transmission and determination of genetic diversity in *M. tuberculosis* populations world-wide. To understand "Emerging strains" of *M. tuberculosis* and their epidemiology and distribution, RFLP studies are considered valuable for the public health aspects of the Vietnamese National Tuberculosis Program.

Sputum smears collected, AFB microscopy performed and *M. tuberculosis* strains were grown on Löwenstein-Jensen slants for 4 to 6 weeks at local hospitals.

PCR was used to detect *M. tuberculosis* DNA and a probe hybridization technique was used for genotyping. DNA was extracted, digested and separated by using horizontal 0.8% agarose gels in Tris-acetate buffer and vacuum blotted onto nylon membranes. For the IS*6110* RFLP, the DNA was hybridized to the 254-bp PCR product of IS*6110* and visualized by a digoxigenin-dUTP labelling and detection kit (Roche Diagnostics GmbH, Mannheim, Germany) in Norwegian Institute of Public Health (NIPH) in Norway.

The sample size of this study was too small to make conclusive recommendations. However, clear indications arise. The East African Indian family appears to represent a well established epidemic and presented an epidemiological pattern indicating recent transmission both within and between urban and rural areas. The Beijing and other families however, appeared to represent isolates of ongoing importation to northern of Vietnam. Multi-drug resistance was not found to be related to any particular *Mycobacterium tuberculosis* family.

Despite the presence of several *M. tuberculosis* lineages and the large genetic diversity in the MTB population presented in northern Vietnam, spoligotyping should be considered valuable screening method for clustering of *M. tuberculosis* isolates and their assignment to known genotypes. Also the affordability reproducibility of the method is in its advantage.

I. INTRODUCTION

Annually, *Mycobacterium tuberculosis* (MTB) caused about 9 million new cases of active tuberculosis (TB) and 2 million deaths. It is estimated that over one-third of the world population (1.9 billion people) was infected with TB and 80% of them are from 22 high burden countries. TB can spread through the air, is contagious, a worldwide pandemic, multi-drug-resistant (MDR) and is the leading infectious disease cause of death among adults (15-59 years) (1;2). Nowadays, TB epidemic is still growing in sub-Saharan Africa which is also closely linked to HIV/AIDS, intravenous drug-users and in developing countries. HIV infection worsen the TB situation by increasing reactivation of latent TB infection as well as rapid progression of new infection in the HIV infected (3;4).

To combat TB, Directly Observed Treatment, Short-course (DOTS) has been adopted as the WHO strategy for the global control of TB. Besides, bacille Calmette-Guérin (BCG) is the only currently available vaccine against TB and widely administered in the WHO Expanded Program for Immunization (EPI). BCG has been used to prevent of severe childhood disease but it has offered no overall protection in adult and a low level of overall protection in children, and not expected to reduce the transmission due to TB (2;5).

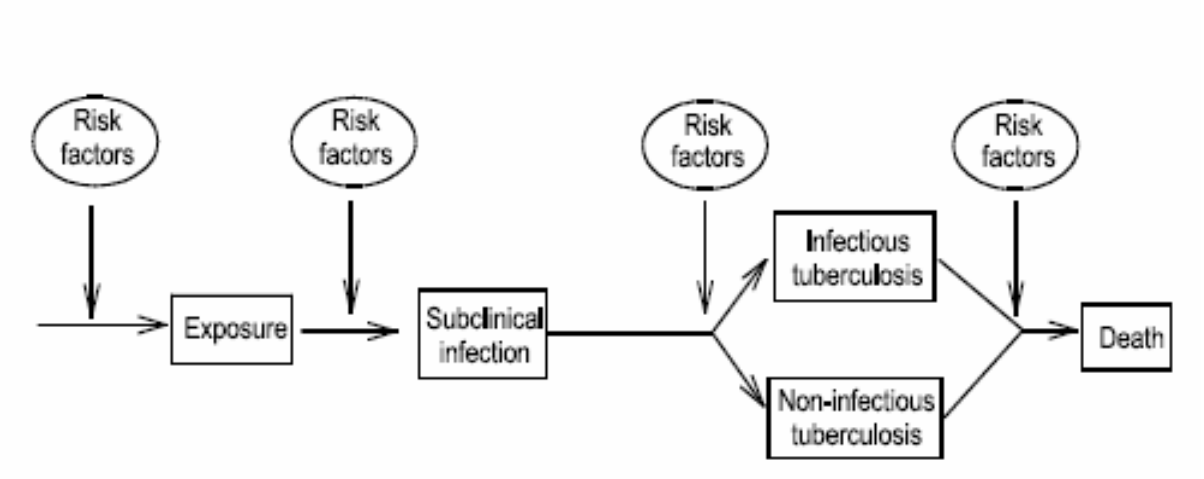
The development of molecular typing techniques and its valuable methods in last decade has contributed to the improvement in studies of infectious diseases in general and TB in particular. Molecular techniques can be used to discriminate exogenous versus endogenous disease, to investigate outbreaks, to study transmission within defined geographic setting, to detect MTB strain acquired MDR (6). DNA fingerprinting of *Mycobacterium tuberculosis* has been shown to be a powerful epidemiologic tool. It is a standardized technique which exploits variability in both the number and genomic position of IS*6110* to generate strain-specific patterns (7). Restriction fragment length polymorphism (RFLP) analysis with the IS*6110* probe is a convenient and reliable method for differentiating *M. tuberculosis* strains (8). A total of 36 potential subfamilies or subclades of MTB complex have been tentatively identified and devided into 8 main families worldwide (9;10) Beijing strain is a new successful strain of *MTB,* representing a high percentage of clinical isolates in South East Asia, South Africa and several locations in the Russia Federation, in newly diagnosed patients Beijing was strongly associated with MDR (11).

Vietnam is a member of high burden countries with approximately 145,000 new cases of TB each year of which about 20,000 deaths are due to TB-related causes (12). Vietnam is the only member of the current group of high-burden countries who has reached the targets for DOTS implementation before the year 2000. TB prevalence was 232 (all cases / 100,000 pop.), incidence 176 (all cases / 100,000 pop.), new TB cases MDR 2.3 %, previously treated TB cases MDR 13% (WHO estimated 2004). Some scientific studies showed that molecular techniques had been conducted in Pham Ngoc Thach hospital, Ho Chi Minh City, however, they were not used as routine activities in the reference laboratories of NTP. On the other hand, those studies had mostly focused on specific genotype, such as Beijing strain by spoligotyping method. Thus, this study research aims to describe the distribution of MTB in the northern of Vietnam. The combination of spoligotyping and IS*6110* RFLP as probe can discriminate exogenous versus endogenous strains as well as demonstrate the transmission between patients living in different geographical areas, and generate strains specific patterns of MTB at the same time. Besides, this study has an opportunity to review sputum smear microscopy technique using bacterial culture as gold standard in screening TB.

II. LITERATURE REVIEW

Epidemiology of TB

2.1 Epidemiology basis of TB: To facilitate the understanding of the relevance of the epidemiology of *tuberculosis* as a basis for implementing a successful national TB control program, a model following the pathogenesis of tuberculosis from exposure to death is useful.



A model for tuberculosis epidemiology, following the pathogenesis of tuberculosis (Hans L. Rieder) (13).

TB suspect: As illness, TB mainly affects the lungs, but it also affect to other parts of the body, such as brain, bones, glands, etc. TB should be suspected if a person has cough for three weeks or more. TB is caused by the germs spread through the air when the infected persons cough or sneeze. People who are infected with do not feel sick, do not have any symptoms, and can not spread TB. They may develop disease at certain time in the future. People with TB can be treated and cured if they seek medical help. Besides coughing, other symptoms of TB may include fever, especially rising in the evening, pain in the chest, loss of weight, loss of appetite, coughing up of blood (14).

2.2 Laboratory:

Sputum smear microscopy for acid-fast bacilli (AFB) remains the first priority for National Tuberculosis Programs (NTP) in high-prevalence countries (13).

Tuberculin Skin Test (TST) usually positive in adults in endemic area of TB. It demonstrates that at some point in the past the person was infected with *Mycrobacterium*. It is, therefore of limited diagnostic value for active TB. A tuberculin reaction of >= 5 mm of in duration is generally regarded as positive (15).

Culture: Bacteriological culture provides the definitive and remains the gold standard for diagnosis of *tuberculosis.* Culture techniques can detect few bacilli (AFB negative cases); diagnosis failures of treatment; provide material for drug susceptibility testing, PCR and DNA fingerprinting (16).

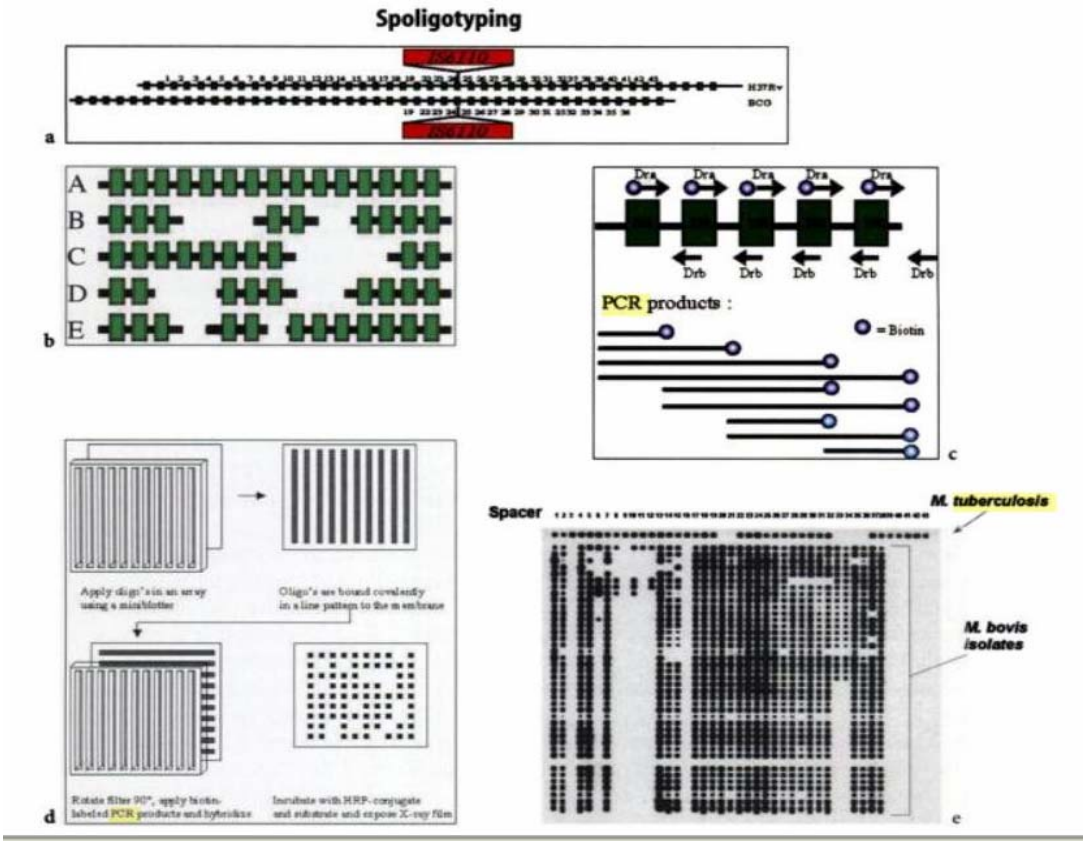
Chest X-ray (CXR) may show evidence of active or healed TB manifestation in the lungs. It may also reveal clustered calcifications in the axilla, suggesting the possibility of lymph node *tuberculosis* in suspected patients (17).

Drug Susceptibility Test (DST): Drug susceptibility compares the growth rate in drug free and drug containing media; detects or measures of the metabolic activity of isolates and genetic mutations using molecular techniques (18). DST is used as a tool for selection of effective regimens to successfully treat tuberculosis patients, evaluate NTP efficiency.

2.3 Molecular Epidemiology

Polymerase Chain Reaction (PCR): Gene amplification methods are designed for the detection of MTB is highly sensitive, especially culture-negative specimens of the disease. A variety of PCR techniques have been developed for the detection of specific sequences of MTB and other *Mycobacterium* species (19). PCR defined segments of DNA can be amplified to microgram quantities from as little as a single template molecule. Although the procedure in some way is deceptively simple, and the reaction can entail complex biochemical interactions, it is in most applications a fast, relatively inexpensive, and easy way to generate sample materials for further analysis (20).

Spoligotyping detects and type MTB complex bacteria, based on polymerase chain reaction (PCR) amplification of a highly polymorphic direct repeat (DR) locus in the MTB genome. Spoligotyping determines causative bacterium and provide epidemiologic information on strain identity. It is useful in surveillance of TB transmission and to measure the interventions to prevent further spread of the disease (21).



(22)

In comparison to other molecular typing techniques such as IS*6110* RFLP, the advantage of spoligotyping is the relatively fast generation of DNA patterns, data can be digitalized therefore simply inter-laboratory comparisons and communication of results. Also it can be performed directly on cell lysates and nonviable bacteria. The limitation is mainly related to its inferior discriminatory power (23).

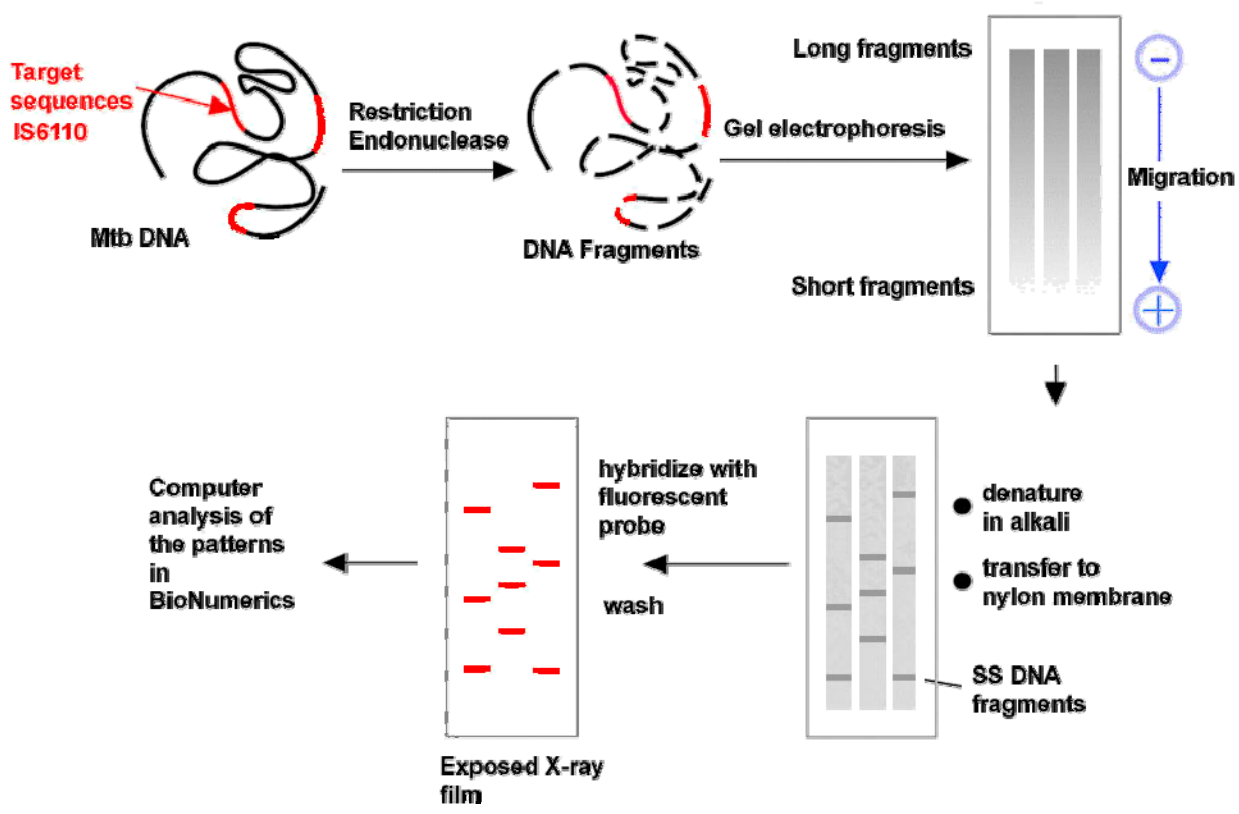
In the "the fourth international spoligotyping database" (10), the population genetics of 39,295 strains from 122 countries were identified, some main TB families/lineages were described. A different in eight regions of the world are described below:

The Central-Asia (CAS) family was essentially localized in the middle-East and Central Asia (21.2%), and preferentially in India (75%).
Haarlem (H) family was distributed 25% of the isolates globally while it dominated in South America by constituting around 50% of the isolates there.
Latin-American-Mediterranean (LAM) constituted 50% of the other haft of the strains from South America.
The "T" family is less well defined and include more than 600 unclassified STs. This lineage is currently stratified into 5 sub-clades (T1-T5).
East-Africa-India (EAI) family was more abundant in South-East Asia, particular in Vietnam and Thai Lan (32%).
X family was highly prevalent in North America (21.5%) and Central American (11.9%) regions.
The Beijing and Beijing related strains represented about 50% of the strains in Far East- Asia and 13% of isolates globally.

Spoligotyping has been shown to exhibit less discriminatory power than IS*6110* RFLP when used for sub-typing of high-copy-number strains but has proven useful for sub-typing IS*6110* low-copy-number strains (24).

Restriction Fragment Length Polymorphic (RFLP) technique may be differentiated by analysis the diversity degree and the distribution of isolates when the DNA is digested with a restriction enzyme. Clinical isolates of MTB studied with IS*6110* – RFLP typing display a high degree of polymorphism and long term stability of RFLP has been demonstrated (8). The IS*6110* RFLP base on the differences between strains in the number and position of copies of IS*6110*, ranging from 0 to 25 in the chromosome (25). Currently, IS*6110* RFLP has been widely used in TB epidemiology in order to answer question related to the transmission, re-infection or reactivation of MTB (26). However, there are some limitations to the IS*6110* RFLP technique. The method is unreliable for typing strains with fewer than 6 copies of IS*6110*. Also the method is laborious, require high tech laboratory equipment and skill, and it represents a difficulty for communication results and inter-laboratory comparative analysis (23;25;27;28).

RFLP analysis diagram (23)



2.4 Transmission and pathogenesis of TB

In natural circumstances, MTB is transmitted by expulsion of exhaled droplets from an infected human individual to an uninfected one. Air-born droplets produced when patients cough, sneeze, speak, sing etc. They may contain tubercle bacilli and are no larger than 2 µm in diameter. These droplets are able to penetrate to the alveoli of the respiratory tract of the uninfected individual (29;30). Tubercle bacilli are necessary, but not sufficient cause TB. While the risk of becoming infected is largely exogenous in nature, determined by the characteristics of the source case, environment, and duration of exposure, the risk of developing TB given that infection has occurred, is largely endogenous, determined by the integrity of the cellular immune system (13).

Alveolar macrophages ingest the MTB and enclose them in phagosomes. If these macrophages are activated, the mycobacteria containing phagosomes fuse with lysosomes,

and the bacteria are killed. If the alveolar macrophages are not activated, the bacilli survive and grow within the phagosomes by altering intracellular compartments in some way to preclude normal maturation to phagosomes or to prevent fusions of the phagosomes to lysosomes (29;31). Ingested tubercle bacilli by other alveolar and monocyte are spread through lymphatic channels to regional hilar and mediastinal lymph nodes and through bloodstream to other organs. The logarithmic phase of bacillary growth is arrested with development of cell mediated immunity and delayed-type hypersensitivity at 2-10 weeks after the initial infection (29;32). Development of specific immunity is usually adequate to limit further multiplication of the bacilli; the host remains asymptomatic; and the manifestations heal. Overall, 10% of people infected with MTB will develop clinical TB sometime during their life (Bass JB et al, 1999). At any time after the initial infection, tubercle bacilli that have spread through the body may begin to replicate and produce disease. The infection of these lymph nodes may progress directly to clinical disease or may become active after years or never at all (33).

2.5 Risk factors for develop active TB

The importance of any risk factor in public health is determined by both the strength of the association and the prevalence of the risk factor in the population (13).

Infection with the human immunodeficiency virus (HIV) and IDU: The risk of TB in IDU varies with the duration of HIV infection, among HIV-infected persons is closely correlated with the number of CD4+ lymphocytes; in HIV-infected persons tuberculosis most often results from the reactivation of latent TB infection (4;34).

Spontaneously healed TB with fibrotic residuals: Persons who had TB which healed spontaneously leaving fibrotic residuals are at increased risk of developing TB again (35).

Age: There are large differences in *tuberculosis* incidence by age. Adolescents and young adults seem to be prone to progression from latent infection to disease while children around the age of 10 years least prone. Variation with stage of maturity is not as likely an explanation of the steady increase in incidence rates among adults up to the age of 60 years. However, there are indications that the risk of tuberculosis following infection increases beyond the age of 60 years (36-38).

Sex: Some recent studies suggest that maturational and hormonal factors may play a role in the risk of tuberculosis and its manifestations (37;39).

Body build: The incidence of TB among person below ideal body weight is higher than among person with normal height and weight (40).

Environmental factors:

Smoking: TB incidence in smoker group higher than non - smoker group was presented in some studies (41).

Chronic ethanol abuse in humans leads to a variety of immuno-modulatory events that can alter resistance to infectious agents, including TB (42).

Nutrition

It is a common notion that malnutrition adversely affects the immune system.

Diet: Vegetarian diet has been identified as a risk factor for TB (43). The active metabolite of vitamin D, 1,25-hydroxy-vitamin D_3, promotes maturation and activation of human monocytes and macrophages, and its inhibitory activity on multiplication of virulent tubercle bacilli in human macrophages has been demonstrated (44).

Medical conditions

The severity of silicosis diagnosed at necropsy was associated with increasing risk of pulmonary TB (45). The incidence of TB among diabetics group was higher than general group (46).

Factors associated with the etiologic agent

Infecting dose effect

Strain virulence: Patients with multi-drug resistant strains will remain infectious for a longer time on average than with patients with fully susceptible organisms, as chemotherapy is likely to be less efficient in rapidly reducing transmissibility. It seems to be confirmed that certain genetic mutations in tubercle bacilli which cause them to become isoniazid-resistant also reduce their virulence in experimental animals (13).

Re-infection and multi-drugs resistant (MDR): The cured patients developed TB disease again with a resistant strain has proven in some previous study. Besides, resistance to anti-tuberculoses drugs can develop not only in the strain that caused the initial disease, but also

as a result of re-infection with a new strain of M. tuberculosis that is drug-resistant (47).

Socio-Economic-Status (SES) impacts TB incidence via both a strong direct of crowding, manifested predominantly in overcrowded setting, and a TB – SES health gradient, manifested at all SES level (48).

2.6 Treatment

To combat TB, WHO has recommended adoption of a new strategy called Directly Observed Therapy Short-course (DOTS). This strategy has five elements, each of which is essential:

- Clear and sustained political commitment by national governments is crucial if basic DOTS and the Stop TB Strategy are to be effectively implemented.
- Case detection through quality-assured bacteriology: bacteriology remains the recommended method of TB case detection, first using AFB microscopy and then culture and DST testing.
- Standardized treatment, with supervision and patient support.
- An effective drug supply and management system.
- Monitoring and evaluation system, and impact measurement.

The DOTS strategy emphasizes completion of treatment and thereby curing of the patient. By doing so, it stops TB at the source, and prevents the spread of the disease, the development of MDR-TB, and complications of TB, relapse and death.

WHO-recommended strategy prolongs survival of patients with AIDS and TB and improves their quality of life. It can be integrated into the general health services and can, therefore, be widely used. The global target for TB control is to cure at least 85% of new smear positive cases and detect at least 70% of such cases. DOTS is the only strategy which has achieved these results on a program basis (49).

DOTS program will have greater effect on TB incidence if it detects cases of infectious and noninfectious TB as soon as possible. The proportion of deaths prevented will generally be greater than the proportion of cases prevented, especially if cure rates have been low in the past and the new program treats smear negative cases (50).

BCG vaccine

BCG vaccines are generally given to protect against TB. Though the WHO now emphasizes BCG's utility in prevention of severe childhood disease (e.g. TB meningitis), the main public health burden of TB is associated with adult pulmonary disease. It is therefore important to consider BCG vaccine efficacy against childhood TB, separate from adult TB.

Booster doses: some studies conducted in Hungary, Poland, Chile and Finland showed that there is no convincing evidence that boosters are effective in preventing TB (51).

2.7 Emerging successful strain

The high prevalence of Beijing strains globally demonstrate that the success of MTB strains type as a human pathogenic (Glynn et al.,2002) (52;53). The genetic of this family was described in 1995, and has been shown to be a highly prevalence in China, Russia, north of America, and other Asian countries. Beijing family strain was significantly related with young TB patients. In addition, the characteristics of Beijing strains that emerging because they have a higher ability to resist the anti-TB drugs and/or BCG vaccination has been shown in many studies (11;47;52-58).

In the case of the Beijing trains, it has shown high prevalence worldwide; resist anti-TB drugs and/or BCG vaccination and wide transmission; relatives to young TB patients as mention above Beijing strain is a new successful strain of MTB (11).

Beijing molecular typing definition: Beijing genotype strains, including W strains, have been characterized by their highly similar multi-copy IS*6110* restriction fragment length polymorphism (RFLP) patterns, deletion of spacers 1 to 34 in the direct repeat region (Beijing spoligotype), and insertion of IS*6110* in the genomic *dnaA-dnaN* locus (59).

The percentage of low-copy number IS*6110* RFLP of MTB was recorded at 26% originated from ASIA while 54% from Central and East Africa (Bauer, J et al 1999), however in this study the MTB strains was not described in genotype families (24;60).

The definition of atypical Beijing is Beijing (spoligodefinition) strains carrying low copies of IS*6110*. It is also based on polymorphism on other genes, but we do not routinely use them for characterization (61).

III. COUNTRY PROFILE

Back ground

http://www.unicef.org/infobycountry/vietnam.html

Vietnam is located in South-eastern Asia, bordering the Gulf of Thailand, Gulf of Tonkin, and South China Sea, China, Laos, and Cambodia.

The climate is tropical in south; monsoonal in north with 4 seasons (Spring, summer, autumn and winter)

The environment: Slash-and-burn agricultural practices contribute to deforestation and soil degradation. Water pollution and over fishing threaten marine life. Groundwater contamination limits potable water supply. Growing industrialization (and population migration) is rapidly in Hanoi and Ho Chi Minh City. The population growth rate is 1.3% (2004 estimated) and the age structure is 29.4% 0-15 years; 65% 15-64 years; 5.6% over 65 years. The Vietnamese population estimated in July 2004 is 82,689,000 (July 2004 est.) (62).

Hanoi profile: The city is located on the right bank of the Red River. Hanoi comprises nine inner districts and five outer districts. Hanoi experiences the typical climate of northern Vietnam, where summers are hot and humid, and winters are relatively cool and dry. The minimum winter temperature in Hanoi can dip as low as 6–7°C (43°F), while summer can get as hot as 38–40 (100-104°F). Hanoi is the largest center of education in Vietnam. It estimated that 62 % of the whole country science cadres are living and working in Hanoi. Because many of Vietnam's major universities are located in Hanoi, students from other provinces wishing to enter university often travel to Hanoi for the annual entrance examination. Hanoi's population is constantly growing, a reflection of the fact that the city is both a major metropolitan area of Northern Vietnam, and the country's political centre. Hanoi estimated population 3,145,300 (2005). Hanoi has the highest Human Development Index among the cities in Vietnam. Though representing only 3.6 percent of the country's population and 0.3 percent of the national territory, Hanoi contributes 8 percent to the national GDP and 45 percent of the Red River Delta's economy. Together with economic growth, Hanoi's appearance has also changed significantly, especially in recent years. Infrastructure is constantly being upgraded, with new roads and an improved public

transportation system. There are about 10 big hospitals and hundreds health care facilities in Hanoi

Thai Binh is a coastal east province in the Red River Delta region of Vietnam, it is about 18 km from Nam Định, 70 km from Hải Phòng, and 110 km from Hà Nội. Thai Binh has a city called Thai Binh city and seven districts. Thiamin estimated population 1,827,000 (2002), including 94.2 % countryside and 5.8% city residence. The climate is as same as Hanoi city. The economic growth rapidly, in 2004 export turnover estimated 78 million USD (increased 22%) while import turnover 57 million USD (increased 20.3%). There were 27,500 tourists visited, including 2,800 foreigners in 2004.

3.1 Healthcare system

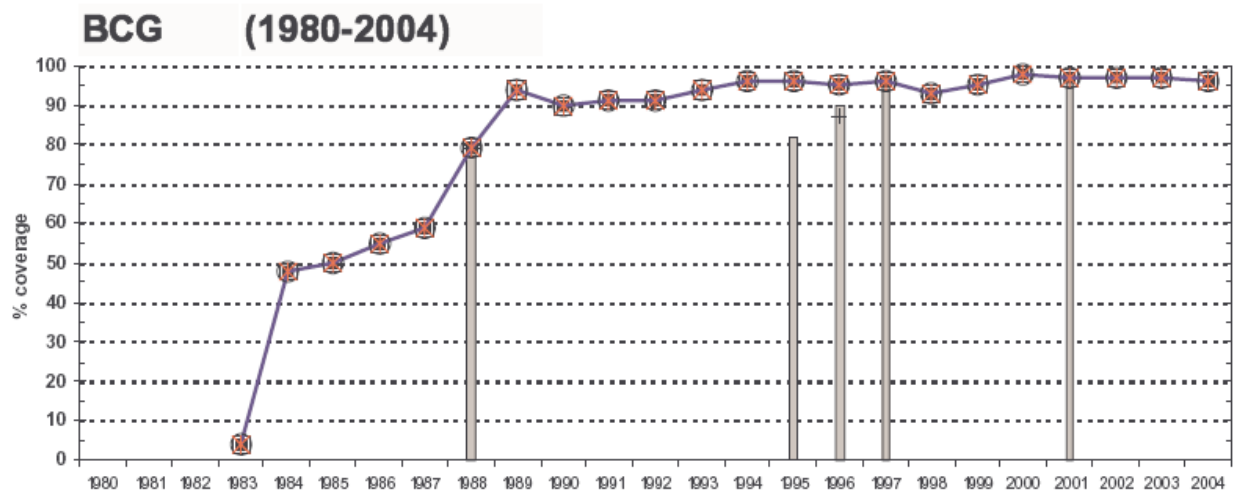
National Tuberculosis Control Program

Anti-TB activities were started in the north as early as 1957 and extended to the south after the country's reunification in 1976. The National Tuberculosis Control Program (NTP) has begun applying the control model of International Union Against Tuberculosis and Lung Diseases (IUATLD) from 1996; this has been successful in many developing countries and is in compliance with the recommendations of the WHO. This model has been deployed and integrated into the network of health services from the commune level to the district and provincial levels. Thus, TB diagnosis and treatment are carried out by commune and district health workers. The TB control program has wide population coverage: In 1998, nearly 99 percent of the Vietnamese population were living in communes and wards covered by the NTP. In 1986, population coverage was only 23 percent. In addition to extensive coverage, the program has been successful in achieving 85 percent cure rate among all detected smear-positive cases and has thus met the objectives set by the WHO (MoH 1999b).

Expanded Program of Immunization

Vietnam began implementing the Expanded Program of Immunization (EPI) on a pilot scale in 1982 and on a nationwide scale in 1985. Since 1993, the country has launched a campaign called the "National Immunization Day," when over 99 percent of children less than five years of age are given two doses of oral polio vaccine (OPV). The EPI program has seen tremendous growth in immunization coverage since 1985 and coverage is now estimated in excess of 90 percent of full immunization for children under one year of age against six vaccine-preventable diseases: diphtheria, tetanus, *pertussis*, poliomyelitis, measles and TB.

Table below shows coverage rates against *tuberculosis* from 1980-2004 (63)



Description of trend

Officially reported data suggest a steep increase in coverage beginning in the 1980's. An EPI Cluster survey in 1988 (Review of the Viet Nam EPI Programme, 1988) confirms the coverage level of almost 80% in 1988. According to officially reported data coverage continues to rise and stablizes above 90% following 1990. Survey data for 1995 and 1996 (MICS, DHS) report slightly lower coverage rates. Card retention, however, is very low in these surveys (13%, DHS). An EPI Cluster Survey in 1998 with card retention of approximately 94% (National Review of the EPI, 1998) confirms reported coverage. The MICS 2000 is not considered because card only data are provided (27% cards seen).

The increased immunization coverage has been associated with a dramatic decline in child mortality from vaccine-preventable diseases (63).

The main challenge to the program is expanding immunization coverage in the mountainous and remote border areas, where the difficult geographical terrain, low incomes, and large ethnic minority populations all contribute to relatively low rates of vaccination coverage (64).

3.2 TB in Vietnam

Vietnam is the only member of the current group of high-burden countries to have reached the targets for DOTS implementation, which were achieved before 2000 and exceeded subsequently. This success was made possible by the effective integration of political commitment, international technical assistance and funding, and efficient community mobilization. Viet Nam has continued to expand the program so as to reach remote population groups who have not had access to TB services, and to strengthen the diagnostic laboratory network. An urgent priority is the development of a national plan for improved TB/HIV coordination. A planned national TB prevalence survey will be of critical importance for measuring the impact of DOTS on the TB epidemic (MoH 1999b).

System of TB control

The National Tuberculosis and Respiratory Diseases Hospital (NTRH), in Hanoi, is

responsible for the activities for all of Viet Nam. *Pham Ngoc Thach* Hospital in Ho Chi Minh City is appointed to supervise the activities for the southern provinces. Each province has a provincial TB centre, under the direction of the provincial health service, which is responsible for the local implementation of the TB control program. The district TB units, directed by the district health centres, coordinate the operation of peripheral TB activities. TB patients are referred to the district health centres from community health posts for sputum examination and initial treatment. An effective national TB laboratory network operates under the supervision of the NTP. There are two reference laboratories (Hanoi and Ho Chi Minh City) that perform culture and DST. Of the 64 provincial TB laboratories, nearly one quarter perform culture. Smear microscopy services are provided by more than 600 district TB laboratories (MoH 1999b).

Surveillance and monitoring

The best estimates of case detection for 2003 (86%) and treatment success for the 2002 cohort (92%) suggest, as in previous years, that Vietnam has exceeded the targets for DOTS implementation. Given the high case detection and cure rates since 1997, a fall in the incidence rate could be expected, reflected in the trend in case notifications. It is unclear why no such decline is visible in the nationally aggregated data, but analysis by province could be more illuminating. Case-notification rates are highest among elderly men and women; suggesting that TB incidence has been higher in the past. It is possible that incidence is not falling perceptibly in Viet Nam because the case detection rate may be lower, and the incidence rate higher, than the WHO estimates. In this context, Vietnam's long planned prevalence survey, improve long diagnosis delay condition and detect emerging successful strains would help to establish the true burden of TB in the country, as well as providing a baseline against which to evaluate the impact of the program on the TB epidemic (65).

OBJECTIVES OT THE STUDY

Primary:

To contribute to the description of the MTB population in Northern Vietnam

Secondary:

To map out the difference in distribution of various MTB strains in urban and rural areas of northern Vietnam.

To evaluate the combination of spoligotyping and IS*6110* RFLP analysis, a Vietnameses MTB population

Evaluate some current risk factor of TB disease in the north of Vietnam

IV. METHODS AND MATERIALS

4.1 STUDY DESIGN AND SET UP: *Cross sectional analytic study*

Descriptive epidemiology is concerned with the distribution of the disease, including consideration of what populations or subgroups do or do not develop a disease, in what geographic locations it is more or less common, and how frequency of occurrence varies over time (66).

Cross-sectional survey or prevalence survey provided information about the frequency and characteristics of a disease by furnishing a "snapshot" of the health experience of the population at a specific time and the data can be used to describe characteristics of individuals with the disease and to formulate hypotheses, but not to test them (66;67). Cross-sectional studies establish association at most, not causality (68).

In an observational analytic study, the investigator simply observes the natural course of event, noting who is exposed and non-exposed and who has and has not developed the outcome of interest (69).

This was a cross-sectional and analytical study conducted in three hospitals (NTRH, HNTRH and TBTRH) located in two different cities namely: Hanoi, capital of Vietnam, over three million inhabitants and Thaibinh province, with less inhabitant (see Country profile part).

In this study, the defined population was from patients under 15 years and more than 40 years of age from Hanoi and rural areas in the northern of Vietnam admitted to the NTRH – HNTRH - TBTRH during the period from September to November 2006 by research team. In order to isolate MTB strains contribution, samples were collected from gastric aspirate (gastric liquid) in hospital of children population and from sputum smear of adult population into the study. The researcher assume that, two above mention age groups differ to BCG vaccine status, in order to make clear the ability of BGC vaccination do or do not protect against emerging TB strains if possible (70).

The samples processing and some of initial laboratory techniques (AFB, culture) were conducted in local hospitals. DST was conducted in NTRH, which is currently a reference

laboratory of NTP. All specimens were heat-killed by temperature and kept in a freezer before transport to NIPH, Norway, where the molecular analyses took place. The National Institute of Hygiene and Epidemiology (NIHE) and Norwegian National Ethical committee approved this study.

4.2 STUDY POPULATION

Study population in the study was recruited from all patients admitted to NTRH, HNTRH and TBTRH from September to November 2006 and divided into 2 groups of under 15 years and over 40 years of age respectively. All of them will be isolated MTB strains from samples collected by gastric aspiration (lavage) or sputum smear technique. Gastric lavage collects the respiratory secretions which are swallowed by children at night (71).

4.3 SAMPLE SELECTION

4.3.1 Sample size

With references from a previous study, conducted in 1998, Beijing genotype was found more frequently among BCG vaccinated than unvaccinated people. However, the association in this study was not significant after adjusting for age (25;57), the percentage of Beijing strains was increased from 41% to 71 % (p>0.05; CI 95%) while the age of patients reduced from 65 to less than 25 (57). The researcher assumes that the percentage of W-Beijing strain appropriate at 80% and 50% among children under 15 years and adult over 40 years of age group respectively during the research study period. How large and how does significant need to find out between the two groups? The following formula has been used for sample size to calculate and determine the significant difference between the proportion of Beijing strain of patients under 15 and over 40 year old (72;73):

$$n = Z^2_{(\alpha,\beta)} x \frac{p_1(1-p_1) + p_2(1-p_2)}{(p_1 - p_2)^2}$$

in which: p_1 is the proportion Beijing cases among total TB patients under 15 years old

p_2 is the proportion Beijing cases among total TB patients over 40 years old

α is significant statistic standard (level), probability getting error type I (reject Ho when it is true), it usually define at 0.1 or 0.05 or 0.01 correlative CI= 90% or 95% or 99%

β is a probability getting error type II (accept Ho when it is false), it usually define at 0.1

Z^2 looks up from table below:

Value of α	**Value of β**			
	0.05	0.1	0.2	0.5
0.1	10.8	8.6	6.2	2.7
0.05	13.0	**10.5**	7.9	3.8
0.02	15.8	13.0	10.0	5.4
0.01	17.8	14.9	11.7	6.6

p1 and p2 were supposed to be 0.80 and 0.50. With 5% significance level and 80% power (then Z-square = 10.5) to detect a difference of 0.30 (or 30%) between the two groups we needed 48 subjects in each group. In all, the number subjects to be included were 96.

The number of TB cases needed in each group (under 15 years and over 40 years of age) = 48
In order to make the sample as large as possible, the **convenient or accidental sampling** is chosen to practice in this study.

4.3.2 Sampling technique

Convenience sampling method was applied in the study because it is relatively easy and inexpensive to conduct. By this way, two age group patients admitted to 3 hospitals were recruited into the study. The period of time to select subjects started from 1st September to 30th November 2006. MTB strains distribution were characterized by molecular analysis and used Bionumeric software combine visualized. All demographic, clinical, laboratory and response to treatment were analyzed by SPSS version 13. A data collection form was used for all patients. Information was collected from patients or children parents/guardians. (Annex 2)

Selection of cases

All *tuberculosis* patients of the specific age groups were recruited into the study after they or

their parents/guardians had express the willingness to participate. The willingness to participate by the patients themselves or parents/guardians was confirmed after the contents of the consent form (Annex 3) was read to them.

TB patient's diagnosis was confirmed by 3 hospitals.

4.3.3 Inclusion and exclusion criteria

Inclusion criteria

All TB patients less than 15 years or over 40 years of age admitted to and confirmed by 3 hospitals from September to November 2006 were eligible for the study.

The MTB strains were collected from the patients before the treatment with anti TB drugs was prescribed.

Exclusion criteria

Patients were not willing to participate to the study.

4.3.4 Data collection

The pre-testing was conducted on 5 parents/guardians (mothers) of TB children less than 15 years of age, in order to test the data collection forms. They were not recruited into the main study after the selection of subjects. The idea was to check if they gave relevant answers to the questions to minimize information distortion.

Seven research assistants were recruited into this study, three from NTRH, two from HNTRH and two from TBTRH. The questions and their meanings were thoroughly explained to the assistants. They were then instructed how to ask questions and how to report what the respondent answered. The assistants practised together to ensure a standardised way of collecting information.

In the process of collecting data, the principal researcher and the assistants checked and qualified data after each day of data collection. Corrections were made as necessary and possible.

The questionnaire (ANNEX 2)

The questionnaire was first in English and was translated into Vietnamese language and

followed a clear and simple questions design. The questionnaire had open-ended and closed questions. There was one questionnaire form in order to collect information from each patient.

Data collection techniques

Interviews

Direct interviews were conducted on hospitalized patients. Interviewers first informed interviewees that participation in the study was voluntary. The interviewers explained the purpose of the study and asked interviewees for their permission to interview and collect strains samples from the patients/themselves. Interviewees were also informed that the information they provided would be handled as confidential and their personal answers would not be known, except by the interviewer and the coordinator of this study.

The interview obtained information about demographic data, symptoms of TB, treatment history and BCG vaccination status.

Laboratory methods

The diagnosis of TB in children remains a difficult one. Children with pulmonary tuberculosis are usually unable to produce sputum, and gastric aspirates remain the procedure of choice for microbiologic confirmation of TB (74). The standard approach to collecting gastric aspirates is to hospitalize the child and collect three aspirates on consecutive mornings. The expense of hospitalization and the variable yield from gastric aspirates discourages the routine collection of gastric aspirate from children suspected to have TB (75). Approximately 10 ml of gastric aspirate were collected into a sterile container in the early morning following an overnight fasting. The aspirates were sent to the laboratory as soon as possible and process within 3 hours of collection.

Samples (sputum and/or gastric liquid) were collected from patients at 3 hospitals in the period of the study. Gastric lavage technique was conducted in NTRH with patients from 0 to 5 years old only, depending on the availability of skilled staff.

4.4 Laboratory

4.4.1 Acid-fast bacillus (AFB) microscopy:

AFB microscopy used in this study as a standard method of Vietnam NTP according to the recommendation in the IUALTD and WHO guides. 3 sputum smears were obtained early morning after rising in the first 3 day of each adult hospitalized patient. Ziehl-Neelsen (containing fuchsin 0.3%) was used as stain method, with 15 minutes of contact after heating. De-staining was used acid alcohol (3% hydrochloric acid in alcohol or 70% alcohol), and 20 sulphuric acid in water. Counterstaining was used methylene blue in a 0.3% concentration for maximum one minute. 3 experienced technicians were selected by head of microbiology department and the author of study, 1 from each hospital was been responsible for microscopic examination. Recording and reporting was done by technician, the IUATLD/WHO scale was used as evaluation tool, in case the ordinary microscopy minimum 1 AFB per 10 field or 10/100 was presented for 1+ result…(76)

4.4.2 Culture:

N-ACETYL-L-CYSTEIN-SODIUM HYDROXIDE (NACL-NAOH) was used in all of three hospitals in this study. The mucolytic agent NACL (used for rapid digestion of sputum) enables the decontaminating agent (NAOH) was used at a lower final concentration of 1%. Sodium citrate is included in the digestant mixture to bind the heavy metal ions which may be present ij the specimen and could in active the acetyl-cysteine (16). Samples were grown up from 3-5 weeks and killed by temperature, then were kept in freezer at (– 20^0C) before transportation to NIPH, Norway.

4.4.3 Drug susceptibility testing (DST):

The Proportion method was used in NRTH only, with Löwenstein-Jensen (LJ) medium. DST protocol was used in NTRH during the period of study according to the guideline of the WHO. Resistance is expressed as the percentage of colonies that grow on critical concentrations of the substances, i.e. 0.2 mg/l for Isoniazid, 2 mg/l for Ethambutol, 4 mg/l for dihydro streptomycin sulfate, and 40 mg/l for Rifampicin if LJ medium has been used. The interpretation will be based on the usual criteria for resistance, i.e. 1% for all drugs. (77).

The LJ medium was used for all the resistance tests. The control (egg) medium without drugs was prepared at the same time as the drug-containing media. The period of validity of the media stored at 48^0C is 2 months.

DST was conducted at NTRH parallel with spoligotyping and RFLP techniques in Norway in the same time.

4.4.4 Molecular typing

PCR: In this study, the terminal region of the MTB genome, where direct repeats are located (namely A; B; C; D etc) will be amplified by primer DRa and primer DRb. Amplification of the spacers are accomplished by using the primers DRa and DRb, which enable one to amplify all spacers between the DR sequences. Only a small amount of template DNA is required. Typically the PCR is performed on 10ng purified chromosomal *mycobacterial* DNA. With minor adaptations, frozen DNA extracts from clinical samples can also serve as templates. DRa is labelled with Biotin, ensuring incorporation of this marker in the final PCR product. The primers of PCR are based on the DR sequences:

DRa: 5' – GGT TTT GGG TCT GAC GAC – 3', biotinylated at 5' end

DRb: 5' – CCG AGA GGG GAC GGA AAC – 3'

Spoligotyping is based on DNA polymorphism present at one particular chromosomal locus, the "Direct Repeat" (DR) region, which is uniquely present in MTB *complex* bacteria. With the method described here, the presence or absence of 43 DR-spacers of known sequence can be detected by hybridization of PCR-amplified DNA to a set of immobilized oligonucleotides, representing each of the spacer DNA sequences. This method will be referred to as *spoligotyping* (from *spacer oligotyping*) (78).

PCR products are hybridized perpendicular to the oligo lines. After hybridization the membrance is incubated in streptavidin peroxidase, which binds to the biotin label on the PCR products. Hybridization signals are detected by the enhanced chemiluminescence (ECL) detection system. The peroxidase present on the streptavidine catalyzes a reaction resulting in the emission of light (79).

Isolation of high molecular weight genomic DNA from *Mycobacteria*

The method of choice is determined by the amount of starting material, the desired amount and purity of the DNA isolated, and the nature of the materials from which the DNA is to be extracted. DNA isolated by most miniprep protocols may contain -RNA contamination which can interfere with DNA digestion and with hybridization of the Southern blots. RNA can be removed either by digestion of the entire sample with DNase-free RNase after

preparation, or at the same time as restriction endonuclease digestion (20).

4.4.5 Molecular fingerprinting of MTB strains isolated using IS*6110* as probe:

IS*6110* RFLP analysis

Extraction of the chromosomal DNA from the MTB isolates and Southern blot experiments were performed according to the standardized protocol of Kristin Kremer et al (Annex 2)(80). DNA was digested with restriction endonuclease *Pvu*II and hybridized with a 245 bp, PCR-amplified DNA probe directed against the right arm of the IS*6110*. The probe was nonradioactively labeled with random primed DNA labeling with digoxigenin-dUTP, alkali-labile and detection of hybrids by enzyme immunoassay (DIG). To facilitate the computer-assisted comparison of the fingerprints, we used the *Pvu*II-digested chromosomal DNA of MTB Mt14323 as a reference in each analysis (annex 2) and an external 1 kb molecular weight marker (81).

The fingerprint patterns of the isolates were compared both by computer-assisted analyses using BioNumerics Version 1.5 software (Applied Maths, Kortrijk, Belgium), and by visual examination. All bands that were found to have similar RFLP patterns by computer analysis were visually compared and classified. A cluster was defined as a group of two or more strains from different patients, whose DNA fingerprints were identical with respect to both number and molecular size of all bands. Strains with unique DNA fingerprints were classified as non-clustered (82).

4.5 Variables and definitions used in this study

Two types of variables were used in the study, namely dependent and independent variables.

Dependent variables

MTB strains were characterized at the Norwegian Institute of Public Health (NIPH) Norway.

Independent variables

The independent variables in the study were regarded as the potential risk factors for TB based on the literature review, including demographic, socio-economic factors, transmission, drug resistant., BCG status… age, sex, smoking, alcohol, TST test etc of both of patients in different geographical (67).

4.6. Data analysis

Data collected were entered into a computer for analysis. SPSS 13.0 software for windows (Apache Software Foundation, release 1 Sep 2004, using LEADTOOLS © 1991-2000, LEAD Technologies, Inc) was used for the analysis of the data.

Numerical variables such as age of patients were entered as they were without being recoded. In the other hand, categorical variables like sex, AFB positive or negative, culture, smoking, alcohol abuse, HIV test, TST test, etc, TB history were entered after being recoded.

Categorization of clinical systems of TB was in 7 groups, no known symptoms, experienced symptoms 1 week, 1-4 weeks, 1-5 months, 6 months – 1year, and over 1 years. The categorization was based on the period of symptoms presenting before the 1st day admitted to hospitals.

Categorization of name of strains of TB confirmed by spoligotyping was filled as they were without recoded.

Percentage, frequencies, cross-table were used as the tools of data presentation in a descriptive study, to given the number or proportion of time that observation occur in the study population as well as present discrete data;

Bivariate analysis was performed to test the association of relative risk and TB, by calculating the OR and 95 % CI, with the statistical significance that was set at the level $p < 0.05$. Multivariate analysis was then used to find out whether (or not) the factors, which were significantly identified in bivariate analysis, remain independently associated with the risk of TB(83).

The fingerprint patterns of the isolates were compared both by computer-assisted analyses using BioNumerics Version 1.5 software (Applied Maths, Kortrijk, Belgium), and by visual examination.

4.7 Ethical consideration.

The researchers have to explain the purpose and benefits of the study to the subjects and ask them for their permission to interview and collect specimens. Participation in the study is totally voluntary. Participants will not be forced or persuaded to participate in the study. Even those who initially accept to participate are free to withdraw in the course of the study if they do not wish to continue. The researchers have to guarantee the anonymity of the participants and the confidentiality of the information they provide.

Since the study is conducted by asking patients recruited to gather information and collecting TB strains samples (gastric lavage or gastric aspirate), the conduct of the study will not pose

any health risk to the participants.

The study must be approved by the Department of International Health, Faculty of Medicine, University of Oslo-Norway and the Ministry of Health (MOH)-Vietnam. The project will be submitted to the two bodies for ethical clearance. Also, permission from NTRH directorate and local authorities (if needed) will be obtained before conducting the study.

4.8 RESEARCH TEAM

In collaboration between Department of Molecular Biology Laboratory-NIHE and 3 hospitals, a research team was established to collect data for the study. It included the principal researcher.

4.9 TIME TABLE

Month	Works
July - August 2006	- Visit and work with NTRH, HNTRH, TBTRH directorate - Recruit researcher assistants - Meet and discuss with all members of the research team to reach a consensus on the study's schedule - Train interviewers and conduct the pre-testing. - Modify the questionnaire as necessary. - Arrange meetings for the research team to discuss and decide solutions to problems occurring in the process.
September	- Collect data and samples
October	- Collect data and samples
November	- Collect data and samples
December 2006	- Collect data and samples, and enter data into the computer
January 2007	- Transport heat killed MTB to NIPH, Norway to analysis
February - April 2007	- Work in NIPH laboratory and collect data
May – June 2007	- Write thesis - Defend thesis

V. RESULTS OF THE STUDY

5.1. Study population

Among the patients, 74 were divided into two groups based on different characteristics of site of residence. A total of 43 (58.1%) patients were from Hanoi (urban) and 31 (41.9%) resided in the surrounding provinces (rural)

Patients were recruited between September and November 2006 at 3 hospitals:

National Tuberculosis and Respiratory Diseases Hospital (NTRH)

Hanoi Tuberculosis and Respiratory Diseases Hospital (HNTRH)

Thaibinh Tuberculosis and Respiratory Diseases Hospital (TBTRH)

Table 5.1: Gender and age group distribution by geographic

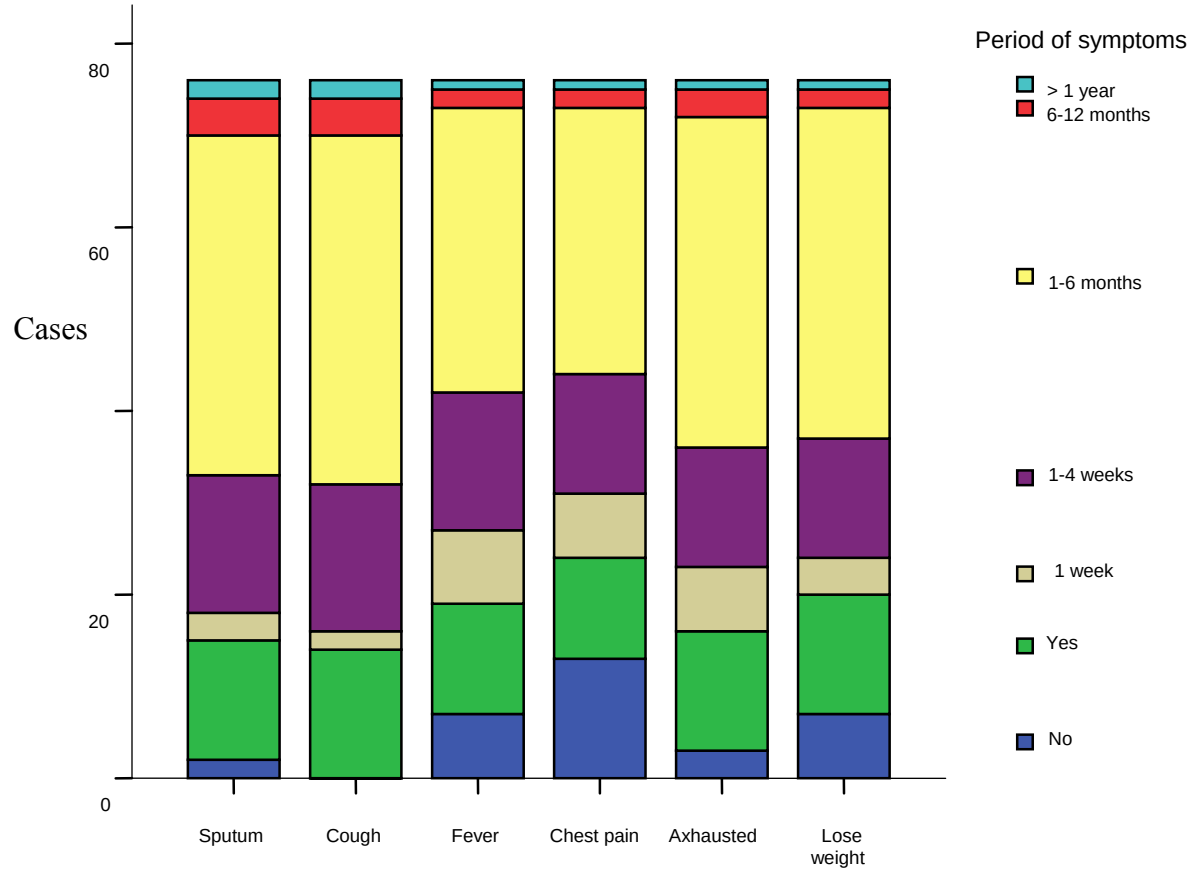
Living place	Gender	Age group		Total		
		Children < 15	Adult > 40	**M**	**F**	
Hanoi	Male	0	36	**36**		
	Female	1	6		***7***	
Total		1	42			**43**
Provinces	Male	2	19	**21**		
	Female	2	8		***10***	
Total		4	27			**31**

Age distribution was presented a big different between adult and children in this study, 69 (93.2%) adults versus 5 (6.8%) children.

5.2 Clinical systems demonstration – Delay to diagnosis TB

The period of clinical symptoms of patients was confirmed in the 1st day of admission to hospital. During the first 1-6 months of clinical symptoms, the patients presented similar symptoms. 47.8% patients recorded sputum symptom during 1-6 month before diagnosis. (47.8% of cough, 37.7% of fever, 39.1% of chest pain, 44.9% of exhausted and 44.9% of lose weight).

Table 5.2: Distribution of cases by clinical symptoms



Distribution of cases by clinical symptoms

5.3 Characteristics of the study samples by AFB and bacterial culture

A total of 74 samples were collected from 3 hospitals inn the time of study. Positive cultures were retrieved from 56 samples (51 belonged to adult and 5 belonged to children group).

Table 5.3: AFB reviewed by using bacterial culture as gold standard

		Culture		**Total**
		Positive	Negative	
Sputum AFB	Positive	54	4	58
	Negative	2	14	16
Total		**56**	18	74

Assessing diagnostic test formula (83)

Indicators were accounted:

AFB false negatives = 2 / 56 = 0.035 AFB false positive = 4 / 18 = 0.222

AFB sensitivity = 54 / 56 = 0.964 AFB specificity = 14 / 18 = 0.778

Positive Predictive Value (PPV) = 54 / 58 = 0.931

Negative Predictive Value (NPV) = 14 / 16 = 0.875

The AFB PPV 93.1% and NPV 87.5% mean that while those who tested positive, almost certainly AFB positive, among those who tested negative, few were AFB positive. In a screening, this finding presented that most people would initially be truly diagnosed as TB.

5.4 Characteristics of adult group:

5.4.1 Demographic and socio-economic characteristic

A total of 69 adult hospitalized patients were recruited in this study, including 42(60.9%) from Hanoi and 27 (39.1%) from provinces during September to November 2006. In each group, the gender distribution was recorded of 36 (85.7%) male and 6 (14.3%) female from Hanoi group versus 19 male (70.4%) and 8 (29.6%) female form provinces group and all of them belong to King ethnic people. The number of female was smaller than male in both Hanoi and province groups.

19 (45.2%) patients of Hanoi were smokers while 13 (48.1%) of provinces and 22 (52.5%) alcohol abuses versus 17 (43.6%) respectively.

Table 5.4.1: Socio-demographic and clinical characteristic of adult group population by geography

		Hanoi (urban)		Provinces (rural)	
		Number	% of group	Number	% of group
Number of patients		42	100.0	27	100.0
Age > 40 years	*Male*	36	85.7	19	70.4
	Female	6	14.3	8	29.6
Ethnic Group	*Kinh*	42	100.0	27	100.0
Smoking		19	45.2	13	48.1
Alcohol		22	52.5	17	43.6
TB contact		5	11.9	2	7.4
BCG history	*Vaccinated*	1	2.4	0	0.0
TB history		16	38.1	8	25.9
Mantoux (TST)	*Positive*	19	45.2	3	11.1
	Negative	23	54.8	24	51.1
Chest X-ray	*Symbolic*	33	78.6	23	85.2
HIV	*Positive*	0	0.0	0	0.0
	Negative	33	78.6	22	81.5
	Not done	9	21.4	5	18.5
AFB	*Positive*	30	71.4	24	88.9
	Negative	12	28.6	3	11.1
Culture	*Positive*	26	61.9	25	92.6
	Negative	16	38.1	2	7.4

5 (11.9%) patients from Hanoi and 2 (7.4%) patients from provinces are defined tuberculosis history in the past by interviewed and health book reviewed. Only one BCG scar (2.4%) was found in Hanoi patient in this study.

Tuberculin skin test (TST) was conducted for all of population in the first day of admission, 19 (45.2%) positive and 23 (54.8%) negative cases were recorded in Hanoi group versus 3 (11.1%) and 24 (88.9%) in provinces group respectively while TST positive was recorded 67.8% and 32.2% negative in the whole of study population.

33 (78.6%) patients in Hanoi and 23 (85.2%) patients in provinces were introduced active or healed tuberculosis lesion in their lungs by health workers (chest X-ray).

33 (78.6%) Hanoi patients and 22 (81.5%) provinces patients were confirmed HIV negative

but the rest population was not conducted this test during study period. 30 (71.4%) Hanoi patients and 24 (88.9%) provinces patients were diagnosed AFB positive at least 1 of 3 sputum samples, finally 26 (61.9%) and 25 (92.6%) positive cultures were collected from their population respectively.

5.4.2 Distribution of samples by Provinces

Among 56 positive *M. tuberculosis* cultures were isolated in Vietnam, 5 samples were isolated from children group, total 8.9% of collection. The other 51 samples were isolated from adult group.

In adult group, 26 (51%) were collected from Hanoi patients and 25 (49%) were isolated from patients residing in rural areas (5 provinces) in the North of Vietnam. Data on distribution of samples by provinces are shown in figure 5.4 as below:

Table 5.4.2: Distribution of samples by Provinces

		Frequency	Percentage
1	***Hanoi***	***26***	***51***
2	Thai Binh	21	41
3	Hai Phong	1	2
4	Nam Dinh	1	2
5	Bac Giang	1	2
6	Thai Nguyen	1	2
	Total	51	100

Hanoi group: The minimum age was 40 years and the maximum age was 79 years representing an age range of 39 years. The median age was 51.5 years.
Provinces group: The minimum age was 41 years and the maximum age was 88 years representing an age range of 47 years. The median age was 53.0 years.
There was no significant difference of age distribution between Hanoi and provinces age group (CI 95%; interquartile range 28 and 26 respectively).

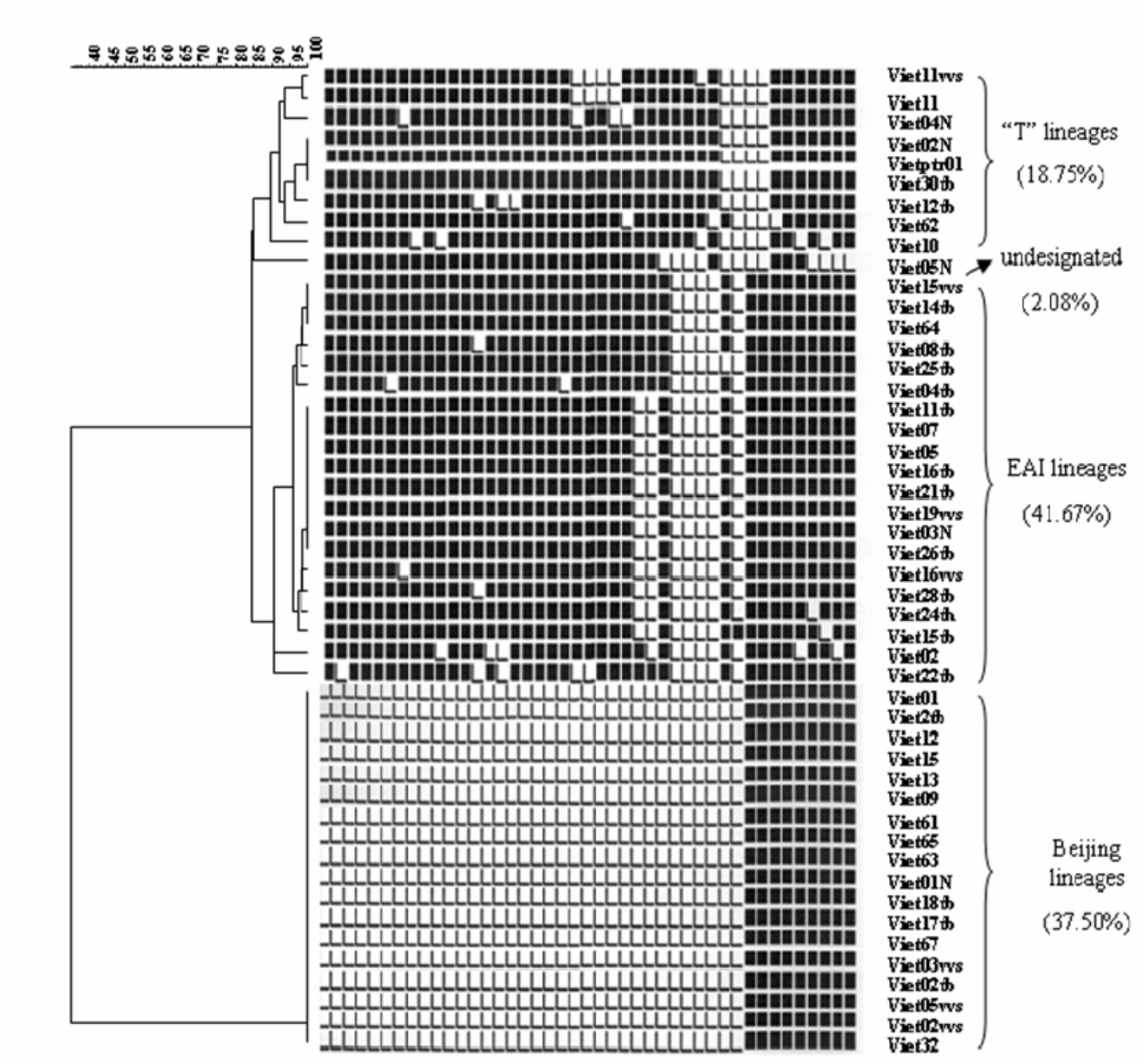
5.5 Molecular characteristic of the study MTB:

A total of 56 samples were identified by spoligotyping and IS*6110* RFLP as probe during the first quarter of 2007.

5.5.1 Spoligotyping results

A total of 48 samples were analyzed and the isolates were divided into two main lineages by spoligotyping. The patterns are depicted in the diagram below:

Table 5.2.1: TB strains distribution were identified by Spoligotyping



The main first group included 18 samples identified as Beijing genotype The MTB Beijing genotype demonstrated hybridization to at least three of the spacers 35 to 43 in spoligotyping and showing an absence of hybridization to spacers 1 to 34 (59).

A sample named Viet01N was recruited from one year children.

In the second group: two globally prevalent lineages; East Africa India (EAI) and the "T" lineage were identified in large numbers.

In subgroup 1: Among the EAI lineages, 8 / 20 samples (from Viet11tb to Viet26tb) were identified as EAI4-VNM genotype. Phylogenetically and geographically this lineage appears specific for Vietnam and surrounding areas (absence of signals 26-27, 29-32 and 34). 3 samples (from Viet15vvs to Viet64) belonged to the EAI-5 (absence of signals 29-32 and 34). The remaining 9 samples in this group were identified as EAI variants (general absence of signals 29-32 and 34) (10). E.g.: a sample named Viet24tb belongs to the EAI1_SOM_EAI4 ((strain number 514, (84))

A sample named Viet03N was recruited from one year child.

***In subgroup 2*:** 7 / 9 samples were identified as "T" lineage (absence of signals 33-36) (10). Brudey et al presented that the "T" familiy (modern TB strains) stayed ill-defined with more than 600 unclassified STs. They were stratified into 5 clades (T1-T5) based on the single-spacer differences. 8 nested clades, with robust spoligotyping-signatures were extracted; with the exception of "Tuscany", their names were built using their proximate upper-clade designation, followed by their presumed geographical specificity T3-Ethiopia, T5 – T1 Russia, T3-Osaka, T4-Central Europe…(10). Besides, 3 of 7 above mention samples (from Viet02N to Viet30tb) have been identified as T1 lineages.

A sample named Viet11 belongs to the Latin-America-Mediterranean 9 lineage (LAM 9) and the sample named Viet11vvs belongs to the H3 LAM9 (ref. 335 – additional database). They have been identified as LAM9 lineages (absence of signals 21-24 and 33-36), but Viet11vvs also carry the characteristics of the H3 lineages (absence of signals 31, 33-36) and this sample was designed of H3LAM9 lineage (10).

2 samples named Viet02N and Viet04N were recruited from one and two years old children.

At last, there was a sample named Viet05N (undesignated) was recruited from a 9 years old children (with previously not described spoligpattern – absence of signals 28-31, 33-36 and 40-43). Its genotype characteristic defined by spoligotyping did not match previously described MTB genotype families. PCR and spoligotyping were performed at 2 different times with the same extracted DNA and gave the same result. The sample was collected from a 1 year old child at the paediatric department of NTRH in December 2006.

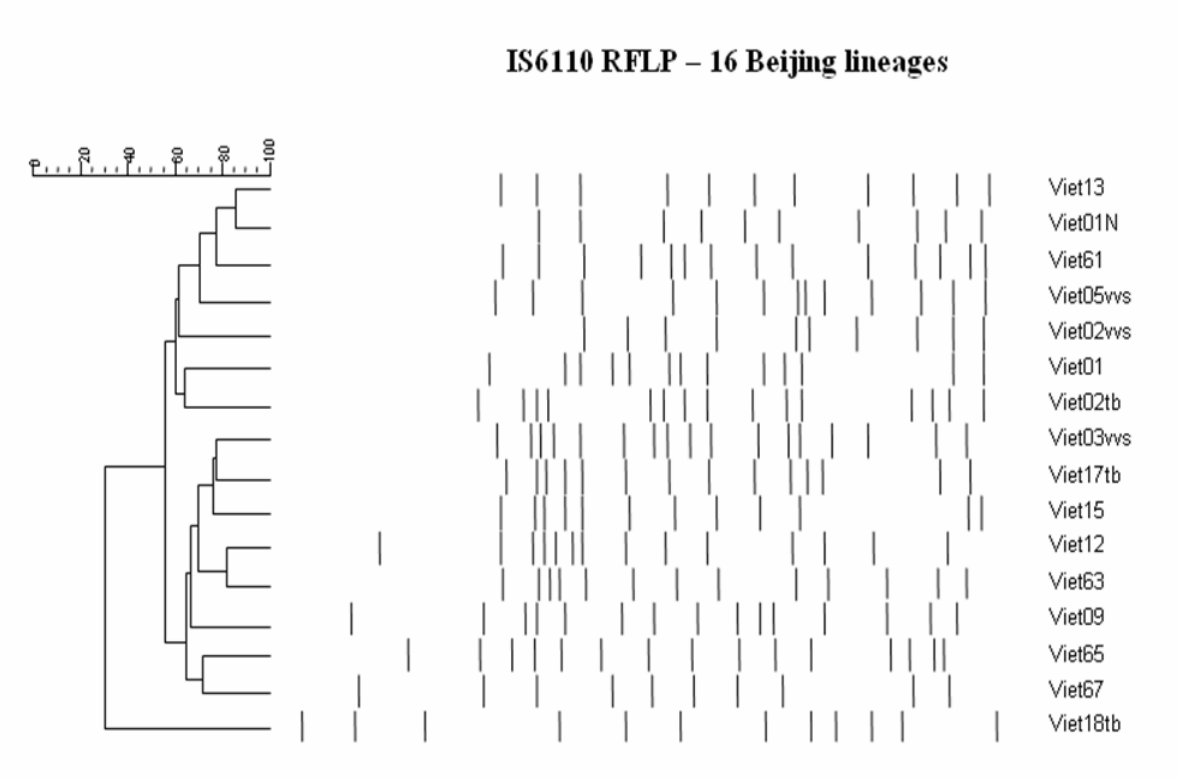
8 samples were not spoligityped (5 from Hanoi and 3 from the countryside). Spoligotyping was attempted two times on all of them, but signals could not be produced, and the examination was discontinued.

5.5.2 DNA finger printing results:

In order to specify the difference of reaction bands of mycobacterium strains, a total of 43 samples (figure 4g) were divided into three groups according to the spoligotyping group results.

5.5.2.1 Beijing group:

The large diversity of the 16 samples assigned to this lineage is presented below. The "IS6110 RFLP_Beijing" shows that all of Beijing isolates were different, despite their identical spoligotypes. The diversity exceeds 80%. Most isolates carry over 10 copies of IS6110 arranged into unique RFLP patterns.



The sample named Viet32 harnours three copies of IS6110 and represents a "low-copy number Beijing isolate" that is referred to as "atypical Beijing" strain.

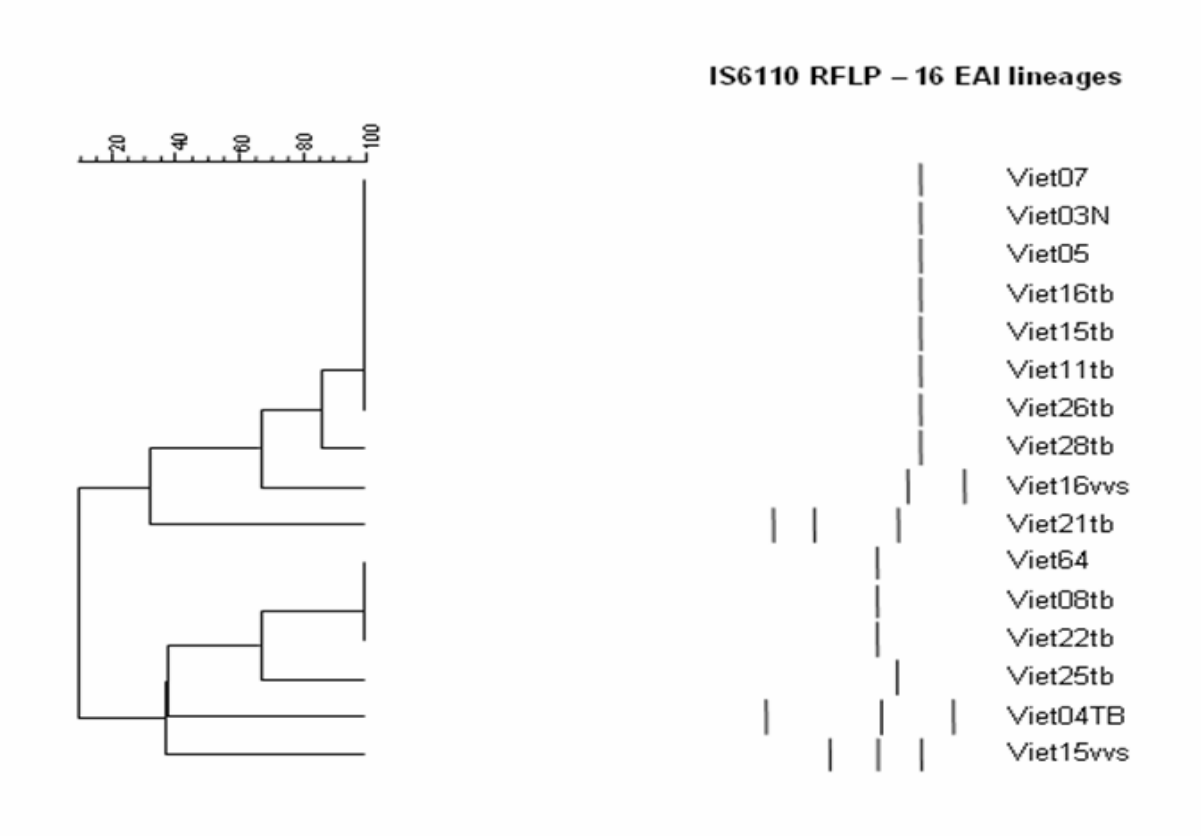
5.5.2.2 East African Indian and other group:

With only 1 isolate carrying unique RFLP pattern among 11 samples carrying identical spoligopatterns of EAI4-VNM (marked * for EAI4-VNM) there were no different level respectively, and EAI-5 lineages (marked ** for EAI-5).

The EAI4-VNM family represents the biggest group in EAI families the EAI family present in Vietnam during the period of the study research.

On the other hand, the remaining of 5 EAI variants has at least 2 isolates presenting unique RFLP patterns and the lineage presented a diversity of up to 80%.

In this group, 9 samples were collected from HNTRH, 4 samples from NTRH and 3 samples from TBTRH.

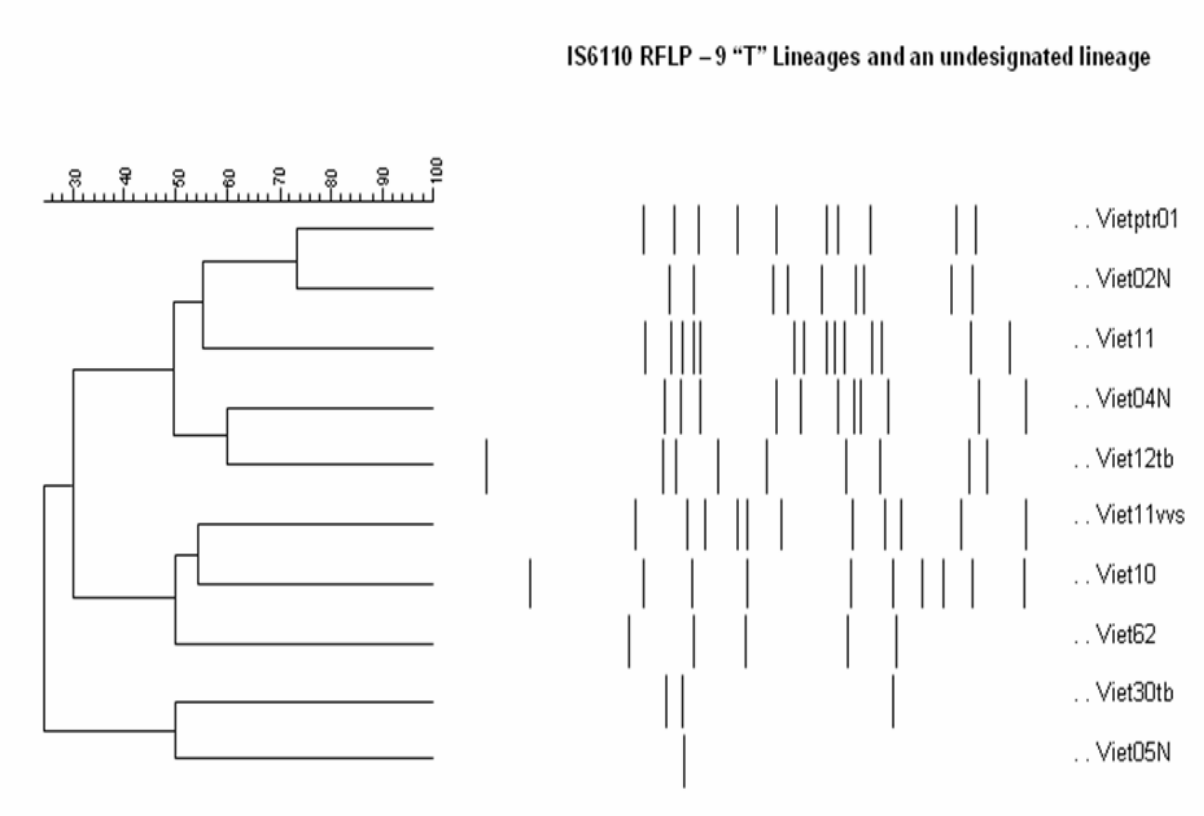


Further to the Spoligotyping results, 4 samples named Viet14tb – 19vvs – 24tb and Viet02 were not got out any isolates into unique RFLP pattern.

RFLP patterns of 16 isolates assigned the EAI lineage, demonstrating a low diversity based on 1-3 copies of IS*6110*.

5.5.2.3 "T" lineage and an undesignated isolate

The isolates assigned to the T-lineage presented unique RFLP pattern. Their number and location of IS6110 copies confirmed a large diversity within this population representing 70%.



The undesignated sample named Viet05N carried a unique RFLP pattern. The single copy of IS6110 was located on DNA fragment of approximately 8 Kbp.

A total of 43/56 PCR products were prepared for both spoligotyping and RFLP studies at the NIPH. These included 38 samples collected from adults and the remaining 5 isolates originated from children (where the gastric lavage technique can be performed only in the pediatric department of NTRH). (Table 5.2.2.4)

Table 5.5.2.4: Distribution of TB strains by IS*6110* RFLP and Spoligotyping

		Spoligotyping						Total
		not done	EAI and variants	Beijing	T variants & others	LAM variants	undesignated	
RFLP	Not done	8	4	1	0	0	0	13
	done	0	16	17	7	2	1	**43**
Total		8	20	18	7	2	1	56

43 samples came form three hospitals in the North of Vietnam: HNTRH, NTRH, TBTRH with 16 / 12 / 15 samples respectively; while the difference in number of samples collected from these above mention hospitals was small. All of 5 samples from children were collected from NTRH. (Table 5.2.2.5)

Table 5.5.2.5: RFLP results distribution by hospitals

		RFLP		Total
		Not done	done	
Hospital name	HNTRH	5	16	21
	NTRH	2	12	14
	TBTRH	6	15	21
Total		13	**43**	56

Geographical strains distribution by spoligotyping

Belonging to the adult group, the strains distribution is shown in detail below, EAI variants were dominant strains in the rural areas while the Beijing strains were abundant in Hanoi city.

Table 5.2.2.6: TB strains distribution by hospital – geographic in adult group

			Hospital name			**Total**
			HNTRH	NTRH	TBTRH	
Hanoi	Spoligotyping	not done	4	1		5
		EAI and variants	4	3		7
		Beijing	8	2		**10**
		T and variants	2	0		2
		LAM variants	1	1		2
	Total		19	7		26
Provinces	Spoligotyping	not done	0	0	3	3
		EAI and variants	0	0	**12**	12
		Beijing	2	1	4	7
		T and variants	0	1	2	3
	Total		2	2	21	25

5.6 Anti TB drugs resistant related to lineages distribution

36 samples (adult and children) were completed in both of spoligotyping and drug susceptibility analysis. 21 (58.3%) samples were demonstrated sensitive with tuberculosis drugs while resistance to any drug was observed in 15 (41.7%) and MDR was observed in 7 (19.4%), (table 4s).

Table 5.6.1: Relationship between TB strains and anti TB drug resistant

Drug resistant	Spoligotyping					Total
	EAI and variants	Beijing	T and variants	LAM variants	undesignated	
Sensitive	10	8	1	1	1	21
1 Drug resistant	2	3	3	0	0	8
2 drugs resistant	1	1	1	0	0	3
3 drugs resistant	1	1	1	0	0	3
4 drugs resistant	0	1	0	0	0	1
Total	14	14	6	1	1	36

Among drug resistance strains (samples were collected before treatment), resistance to at least 1 drug was observed in 8 (53.3%), resistance to 2 drugs was observed in 3 (20%), resistance to 3 drugs was observed in 3 (20.0%) and resistance to 4 drugs was observed in 1(6.7%) .

In adult group:

16 samples were done in susceptibility analysis belong to Hanoi while 15 samples from provinces group, among them 6 of any drug resistance belonged to Hanoi were defined versus 7 belonged provinces group. (Table 4t)

In general, tuberculosis bacterium strains were defined in this study resistant to isoniazid (H) and streptomycin (S) more than rifampicin (R) and ethambuton (E) in both groups.

The main difference between Hanoi and provinces group was presented in EAI family: No drug resistance strain was observed in Hanoi versus 3 of any drugs resistance strains in provinces, (H: 1, HS: 1, HSE: 1)

Table 5.6.2 TB strains and anti TB drug resistant by geographical

Living place			**Spoligotyping**				**Total**
			EAI and variants	Beijing	T and variants	LAM variants	
Hanoi	MDR	Sensitive	5	4	0	1	10
		H	0	1	0	0	**1**
		HRSE	0	1	0	0	**1**
		HS	0	1	1	0	**2**
		S	0	1	1	0	**2**
	Total		*5*	*8*	*2*	*1*	*16*
Provinces	MDR	Sensitive	5	3	0		8
		H	**1**	0	1		**2**
		HRS	0	1	0		**1**
		HS	**1**	0	0		**1**
		HSE	**1**	0	1		**2**
		S	0	1	0		**1**
	Total		*8*	*5*	*2*		*15*

5.7 Analysis results children group

There were only 5 children recruited during the period of study. 1 (20%) of them belong to Hanoi and 4 (80%) belongs to provinces. 2 boys and 3 girls from 1-9 years old were collected specimens from gastric lavage respectively. All analysis techniques were conducted successfully in general and DST test in particular with these samples.
It was defined that, M. bacterium in children resistant to only streptomycin (S) in the period on study.

Table 5.7:1 Relationship between MTB strains and anti TB drug resistant by geographical

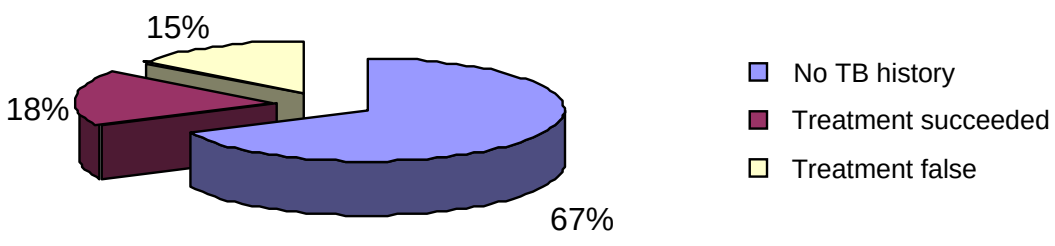
Living place			**Spoligotyping**				**Total**
			EAI and variants	Beijing	T and variants	undesignated	
Hanoi	MDR	S			**1**		1
	Total				1		1
Provinces	MDR	Sensitive	0	1	1	1	3
		S	**1**	0	0	0	1
	Total		1	1	1	1	4

The characteristics of spoligotyping and RFLP techniques of each samples presented in details in the part 4.2.1and 4.2.2 were shown:

A child 9 years old, named Viet03N resistance to streptomycin, was belonged to EAI 4 VNM family by spoligotyping (marked *) and one isolate presented unique RFLP patterns, but was recorded no TB history contact.

Two thirds of the patients (67%) presented no known *tuberculosis* history, while 18% had experienced treatment falure and 15% had treatment success in the past.

Table 5.7.2: Tuberculosis history breakdown



5.8 Multi-bivariate analysis of some risk factors associated with culture positive individuals

Culture positive individuals were significant related to alcohol abuse (OR, 1.429; 95% CI, 0.49-4.20). Alcohol remained strongly associated with MTB infection when controlled by other factor (OR, 2.168; 95% 0.22-2.53). Most patients over 64 year old were significantly more likely to be MTB infected as compared to younger group [odd ratio (OR), 1.677; 95% CI, 0.52-5.43]. When controlled for potential confounding factors, this association disappeared though (OR, 1.793; 95% CI, 0.44-7.37). Considering the gender of population, culture positive individuals occurred significant in male rather than female, (OR, 1.795; 95% CI, 0.51-6.31). Gender remained strongly associated with MTB infection when controlled by other factor (OR, 1.793; 95% CI, 0.44-7.37). Being a TST negative carried a 1.2 time risk of being MTB infected (OR, 1.289; 95% CI, 0.37-4.42). It seems that smoking was not related to risk factor of culture positive infected (OR, 0.456; 95% CI, 0.12-1.65).

Table 5.8: Socio-demographic and other characteristics of MTB culture positive

	Culture		%	OR	95% CI	OR^2	95% CI	p-value
	Positive	Total						
Age category								
> 64	20	25	80.00	1.677	0.52-5.43	0.98	0.96-1.04	0.932
40 - 64	31	44	70.45	1				
Sex								
Male	42	55	76.36	1.795	0.51-6.31	1.793	0.44-7.37	0.419
Female	9	14	64.29	1				
Smoking								
Yes	22	32	68.75	0.607	0.21-1.79	0.456	0.12-1.65	0.271
No smoking	29	37	78.38	1				
Alcohol								
Alcohol abuse	30	39	76.92	1.429	0.49-4.20	2.168	0.22-2.53	0.226
No	21	30	70.00	1				
TST								
Negative	35	47	74.47	1.094	0.35-3.43	1.289	0.37-4.42	0.686
Positive	16	22	72.73	1				

OR, crude odd ratio; OR^2, adjusted odd ratio

VI. DISCUSSION

Strengths of the study

In a cross sectional study, we could easily to collect exactly subject and can link the data items person by person. It provides the status of an individual with respect to the presence or absence or relationship between exposure and diseases at the same point in time. Cross sectional study is most useful for description. Base on the questionnaire and hospitalized patient record we can measure the frequencies of diseases and examine a lot type of variables and establish where the risk factor or disease came first. Convenience sample size allowed collect the number of samples as much as possible in a limited time.

The combination of spoligotyping and IS*6110* RFLP as probe techniques can discriminate exogenous versus endogenous disease, investigate outbreak, study transmission between different geographic setting, and detect MTB strains acquired MDR. More over, those techniques conducted in a standardize laboratory in Norway, that ensure the quality of outcome.

Trainings for research assistants and pre-testing conducted before data collection ensured a standardized way of colleting information and quality of data as well as recruitment patients. All of research assistants are doctors and nurse, experienced in clinical medicine and in doing research.

Vietnamese language used as mother tongue for interview also reduced misunderstanding between interviewers and interviewees.

Limitations of the study:

The sample size was too small to make conclusions on epidemiology in this study. Besides, the bias could not be controlled and was not useful for testing a hypothesis.

Delay to start is the main limitation, lack of skilled staff and low quality infrastructure of laboratory at the beginning of the study. Lack of skilled human resource in conducting gastric lavage technique in TB hospitals, there was only a skilled doctor recruited for this

activity during the time of study. Missed children on the way transportation to the hospital where gastric lavage technique fulfilled. Those reasons reduced the sample size of children group.

Molecular techniques used very few and were not routine activities in NTP system in current time as well as gastric lavage operation.

Results of the study

It is widely recognized that TB is coming back worldwide. TB is a high burden disease especially in developing countries and a major cause of death in adult in Vietnam in particular.

The main finding of this study to map out the difference of MTB strains distribution between urban and rural areas and become a reference for comparative studies in the near future. The power of study is not enough to demonstrate a scientifically evidence, but arise clear indications of currently different characteristics of MTB in urban versus rural areas respectively and may make up a small picture of molecular epidemiology of MTB in current time in the northern of Vietnam.

The combination of spoligotyping and DNA fingerprinting techniques in this study will be a valuable pre-testing for the author in working in TB areas while molecular analysis is still not routine in laboratory system of Vietnam NTP.

The percentage of female presenting to health care services with TB symptoms was lower than male in both of urban and rural areas which was consistent with previous studies in Vietnam and Ghana (85;86). In this study, the percentage of female was 23% versus 77% of male in adult group. According to WHO report in 2006, some epidemiological studies showed the incidence rates failing among older adult (especially women) but rising among younger (especially men) (87). The main reason could be the fear of social isolation from family or community, stigma, gender roles, socioeconomic and level of education described in Vietnam in 1999. However, the mean total delay to TB diagnosis was shown no significant difference between men and women (Long et al; Hudelson et al) (88;89).

In general, 1-6 months admission's to hospital after first symptom demonstrated in this study, 48.7% of sputum symptom, 50% of cough, 40.8% of fever, 38.2% of chest pain, 47.4% of exhausted and 47.4% of lose weight in this study in comparison with 13.3 weeks (4 months) (95% CI, 11.5, 15.1 Long et al) . In comparison to previous study conducted in Ho Chi Minh City, in Vietnam from April 1997 to January 1998 among 801 patients (90), delay to analysis decreased from 81% to 50%-38.2%. The out come of this study was not focus and a limitation to identify the different of delay to diagnosis TB patient between urban and rural, but some previous studies specified longer delay in rural than urban areas (91). However, EAI family well established an epidemic in northern of Vietnam during the period of study, and those MTB strains dominated in rural areas.

TST 67.8% positive and 32.2% negative in adult group were a little bit different to 72% and 28% in a previous study in 2003-2004 (Jiri Homolka et al) (92), but only 1.4% of them were confirmed BCG vaccinated by research members. This identification in most adult patients who has been previously infected MTB without BCG efficiency, TST should be considered using as screening test for non BCG vaccinated population in high prevalence countries of TB or not, to avoid increase infection remains question in this study. (TST may increase MTB infection in the non BCG vaccinated population even in low prevalence countries if used as a screening test for TB) (93).

Delay to analysis was a risk factor for transmission of MTB in the community, especially relating to sputum smear positive (94). AFB microscopy was the first diagnosis technique in community level of Vietnam NTP according to the recommendation in the IUALTD and WHO guides. TB prevalence was 92.653 (10/10.000 of population), AFB positive incidence was 59.8%. To review the AFB test which has been used as the first priority for NTP using bacterial culture as a gold standard for diagnosis of tuberculosis (17;95), the sensitive of AFB test was accepted at 96.4% while the specificity in this study 77.8% versus 98-99% of recently studies in high-prevalence countries (96;97). The PPV 93.1% and NPV 87.5% mean that while those who tested positive, almost certainly positive, among those who tested negative, few were positive. In a screening, this finding presented that most people would initially be truly diagnosed as *tuberculosis*. If this study has enough power, AFB test will still be the first priority for NTP for diagnosis *tuberculosis* in study located in current time while MTB culture remains the gold standard for diagnosis of TB.

Among study population, 78.4% AFB positive versus 21.6% AFB negative were similar to

the average percentage of Hanoi and Thai Binh indicators 80.6% and 19.4% respectively, (Vietnam Heath statistics book 2003).

In general, the molecular epidemiology in northern Vietnam is dominated by two main MTB families:

EAI family was the biggest group of lineages at 41.7% of the total of samples, and 65% of those strains belonged to provinces. There is a well established of EAI epidemic, carrying unique RFLP pattern and the spoligotyping pattern and spoligotyping pattern identical for EAI4-VNM (marked * in the spoligotyping diagram). Identical spoligo and RFLP patterns would generally indicate an ongoing active transmission of MTB. The low number of IS6110 copies however, urges caution in order to make such conclusions since isolates carrying less than 5 copies of IS6110 might not represent recent transmission although their RFLP pattern is identical. The EAI-4 and EAI-5 families with the same characteristic IS6110 copies belong to each family respectively, indicated that EAI epidemic as mentioned above in which Hanoi contributed 3/8 of EAI-4 and 1/3 EAI-5 in total. Is there any transmission between urban and rural areas when this study was conducted remained questionable.

Beijing family was a second main group and contributed 37.5% compare to 46.2% in similar age group (Anh DD et al 2000) and 61% of those strains belonged to Hanoi city. The percentage of Beijing lineages was low in this study because they were mostly isolated from adult group. Beijing isolates were separated into unique RFLP pattern despite their identical spoligotyping pattern. This indicated an older epidemic or importation and not a recent transmission / outbreak.

20.8% of lineages belonged to “T” family (7 / 10), LAM family (2 / 10) and 1 undesignated lineages.

The general characteristics of Beijing, “T” group presented a large diversity. This might present ongoing importation of these lineages. Although it must be emphasized that the current population is small and does not represent the complete epidemiological picture in northern of Vietnam. However few isolates were closely related to other isolates within this population, such observations are generally characteristic for populations that have not evolved locally.

Anti TB drug resistance: among 74 samples of this study, resistance to any drug was observed in 15 (20,3%) versus 26.3% ; resistance to isoniazid 10/74 (13.5%) versus 16.6%; resistant to Rifampicin 2/74 (2.7%) versus 2.0%; resistance to Ethambuton 3/74 (4.1%) versus 1.1%; resistance to Streptomycin 10/74 (13.5%) versus 19.4% respectively. Besides, among 24 treated patients in this study; any drug resistance was 5/24 (20.8%) and MDR was 2/24 (8.3 %) versus and 62.9% and 23.2% (Huong et al), but the sample size in this study was too small to make any conclusion about the difference of these figures (98).

In adult patient group, the difference arises between urban and rural group if the combination of molecular epidemiology with MDR is made. The main difference between Hanoi and provinces group was presented in EAI family: No MDR in Hanoi versus one drug resistance and two MDR strains were defined in provinces, (Viet25tb resistance H, Viet16tb resistance HS and Viet 11tb resistance HSE). EAI-4 VNM and EAI-5 families in northern Vietnam present the same characteristics in molecular and MDR epidemiology as sensitive with anti-TB drugs (excluded Viet11tb and Viet16tb resistance at least 1 drug). It seems that, the cross transmission in the northern Vietnam occurred during the time of study.

TB risk factors in adult population of this study associated between culture positive or active TB with alcoholism (p=0.226) and male (p=0.419), however the limitation of sample size make difficult to conclude a significant evidences. Age and smoking were less relationship with TB risk factors.

21.4% of Hanoi and 18.5% of rural patients unconfirmed with HIV test result in this study, remain as a remarkable point in HIV/TB control activity.

As the children population was too small and is presented here only as additional information, no dominant TB family related to the geographical difference was found. TB transmission occurred in a 9 years old child from rural area, belonged to EAI4 and resistant to Streptomycin. 2 strains belonged to “T” family (including 1 resistant to Streptomycin), and 1 strain belonged to Beijing family. The last strain was an undesignated strain and no examination was followed.

VII. CONCLUSION AND RECOMMENDATION

CONCLUSION

Despite the presence of several M. tuberculosis lineages and the large genetic diversity in the M. tuberculosis population presented in northern Vietnam, spoligotyping should be considered as a valuable screening method for clustering of M. tuberculosis isolates and their assignment to known genotypes. Also the affordability reproducibility of the method is an advantage.

Further research into the presence of putative emerging strains and the risk of transmission of specific M. tuberculosis lineages seems to be necessary in order to improve the effectiveness of the National Tuberculosis Program in Vietnam.

The sample size of this study was too small to conclude what strains are emerging. However, the variation within each of the major strains/families and the urban-rural difference make it possible to hypothesize that the East Africa India family is well established which seems to present a recently cross transmission between urban and rural areas while Beijing and other families continue to be imported in northern of Vietnam.

RECOMMENDATION

- ✓ To consider spoligotyping method for screening MTB as a standard method in all reference laboratories of NTP provinces

- ✓ To focus on education of public on risk of infection.

- ✓ To maintain Immunization and monitoring.

- ✓ To make decision makers (health and politicians) aware of the potential risk and danger of MRD TB in Vietnam

Reference List

(1) WHO. 2006 Tuberculosis Facts. 2006.

(2) Narain JP, Lo YR. Epidemiology of HIV-TB in Asia. Indian Journal of Medical Research 2004; 120(4):277-289.

(3) Narain JP, Raviglione MC, Kochi A. Hiv-Associated Tuberculosis in Developing-Countries - Epidemiology and Strategies for Prevention. Tubercle and Lung Disease 1992; 73(6):311-321.

(4) Selwyn PA, Hartel D, Lewis VA, Schoenbaum EE, Vermund SH, Klein RS et al. A Prospective-Study of the Risk of Tuberculosis Among Intravenous Drug-Users with Human Immunodeficiency Virus-Infection. New England Journal of Medicine 1989; 320(9):545-550.

(5) WHO reports 10 million TB patients successfully treated under "DOTS" 10 years after declaring TB a Global Emergency. 3-24-2003.

(6) Small PM, Mcclenny NB, Singh SP, Schoolnik GK, Tompkins LS, Mickelsen PA. Molecular Strain Typing of Mycobacterium-Tuberculosis to Confirm Cross-Contamination in the Mycobacteriology Laboratory and Modification of Procedures to Minimize Occurrence of False-Positive Cultures. Journal of Clinical Microbiology 1993; 31(7):1677-1682.

(7) Vanembden JDA, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B et al. Strain Identification of Mycobacterium-Tuberculosis by Dna Fingerprinting - Recommendations for A Standardized Methodology. Journal of Clinical Microbiology 1993; 31(2):406-409.

(8) Tuyen LTK, Bach KH, Ho ML, Le NV, Nguyen TN, Chevrier D et al. Molecular fingerprinting of Mycobacterium tuberculosis strains isolated in Vietnam using IS6110 as probe. (vol 80, pg 75, 2000). Tubercle and Lung Disease 2000; 80(3):171-172.

(9) Filliol I, Driscoll JR, van Soolingen D, Kreiswirth BN, Kremer K, Valetudie G et al. Global distribution of Mycobacterium tuberculosis spoligotypes. Emerging Infectious Diseases 2002; 8(11):1347-1349.

(10) Brudey et al. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbioly 2006, 6:23 . 6-23-2006. licensee BioMed Central Ltd.

(11) Gunnar Bjune. Tuberculosis in the 21st century: an emerging pandemic? Norsk Epidemiologi 2005 2007; Norsk Epidemiologi 2005; 15 (2): 133-139.

(12) Global Health Reporting organization. The Role of HealthCommunication in Vietnam'sFight Against Tuberculosis, September 2004. 2007. Global Health Reporting organization. 2004.

(13) Hans L.Rieder. Epidemiologic Basis of Tuberculosis Control. IUATLD 2007.

(14) What Is Tuberculosis and how does it spread? Available in URL: http://w3.whosea.org/tb/tb.htm

(15) CDC, Dvision of Tuberculosis Elimination (DTBE). Core Curriculum on Tuberculosis
Chapter 4 - Testing for TB Disease and Infection Tuberculin Skin Testing. 2000.

(16) WHO/TB/98.258. Laboratory services in tuberculosis control, Part III: Culture. 1998.

(17) What Is Tuberculosis and how does it spread? WHO SEA organization 2007.

(18) Kim SJ. Drug-susceptibility testing in tuberculosis: Methods and reliability of results. European Respiratory Journal 2005; 25(3):564-569.

(19) Tewari M, Shukla HS. Breast tuberculosis: diagnosis, clinical features & management. Indian J Med Res 2005; 122(2):103-110.

(20) Hoelzel. Molecular genetic analysis of populations - a pactical approach second edition. 2007. Oxford University Press.

(21) Gori A, Bandera A, Marchetti G, Esposti AD, Catozzi L, Nardi GP et al. Spoligotyping and Mycobacterium tuberculosis. Emerging Infectious Diseases 2005; 11(8):1242-1248.

(22) edited by M.Monir Madkour. Molecular Epidemiology of Mycobacterium bovis. Tuberculosis. 2004: 80-83.

(23) Frances Jamieson. The Laboratory Diagnosis of Tuberculosis… So you have a confirmed diagnosis of TB (or not!) …now what? 2007.

(24) Bauer J, Andersen AB, Kremer K, Miorner H. Usefulness of spoligotyping to discriminate IS6110 low-copy-number Mycobacterium tuberculosis complex strains cultured in Denmark. Journal of Clinical Microbiology 1999; 37(8):2602-2606.

(25) Nguyen LN, Gilbert GL, Marks GB. Molecular epidemiology of tuberculosis and recent developments in understanding the epidemiology of tuberculosis. Respirology 2004; 9(3):313-319.

(26) Sola C, Filliol I, Legrand E, Rastogi N. Recent developments of spoligotyping as applied to the study of epidemiology, biodiversity and molecular phylogeny of the Mycobacterium tuberculosis complex. Pathologie Biologie 2000; 48(10):921-932.

(27) Dale JW, Al Ghusein H, Al Hashmi S, Butcher P, Dickens AL, Drobniewski F et al. Evolutionary relationships among strains of Mycobacterium tuberculosis with few copies of IS6110. Journal of Bacteriology 2003; 185(8):2555-2562.

(28) van Soolingen D. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. Journal of Internal Medicine 2001; 249(1):1-26.

(29) Dannenberg AM. Pathogenesis of Pulmonary Tuberculosis. American Review of Respiratory Disease 1982; 125(3):25-30.

(30) Clark-Curtiss JE, Haydel SE. Molecular genetics of Mycobacterium tuberculosis pathogenesis. Annu Rev Microbiol 2003; 57:517-549.

(31) Clemens DL, Horwitz MA. Characterization of the Mycobacterium-Tuberculosis Phagosome and Evidence That Phagosomal Maturation Is Inhibited. Journal of Experimental Medicine 1995; 181(1):257-270.

(32) Leung AN. Pulmonary tuberculosis: The essentials. Radiology 1999; 210(2):307-322.

(33) Bass JB, Farer LS, Hopewell PC, Jacobs RF, Snider DE. Diagnostic Standards and Classification of Tuberculosis. American Review of Respiratory Disease 1990; 142(3):725-735.

(34) van Asten. Tuberculosis risk varies with the duration of HIV infection: a prospective study of European drug users with known date of HIV seroconversion. Official Journal of the International AIDS Society 2003; AIDS:Volume 17(8)23 May 2003pp 1201-1208 .

(35) Falk A, Fuchs GF. Prophylaxis with Isoniazid in Inactive Tuberculosis - Veterans Administration Cooperative Study-12. Chest 1978; 73(1):44-48.

(36) Stead WW, Lofgren JP. Does the Risk of Tuberculosis Increase in Old-Age. Journal of Infectious Diseases 1983; 147(5):951-955.

(37) Comstock GW, Livesay VT, Woolpert SF. Prognosis of A Positive Tuberculin Reaction in Childhood and Adolescence. American Journal of Epidemiology 1974; 99(2):131-138.

(38) Epidemiologic Basis of Tuberculosis Control. IUATLD 2007.

(39) Rieder HL. Tuberculosis in An Indochinese Refugee Camp - Epidemiology, Management and Therapeutic Results. Tubercle 1985; 66(3):179-186.

(40) Snider DE. Tuberculosis and Body Build. Jama-Journal of the American Medical Association 1987; 258(22):3299.

(41) Yu GP, Hsieh CC, Peng J. Risk-Factors Associated with the Prevalence of Pulmonary Tuberculosis Among Sanitary Workers in Shanghai. Tubercle 1988; 69(2):105-112.

(42) Potula R, Haorah J, Knipe B, Leibhart J, Chrastil J, Heilman D et al. Alcohol abuse enhances neuroinflammation and impairs immune responses in an animal model of human immunodeficiency virus-1 encephalitis. American Journal of Pathology 2006; 168(4):1335-1344.

(43) Strachan DP, Powell KJ, Thaker A, Millard FJC, Maxwell JD. Vegetarian diet as a risk factor for tuberculosis in immigrant south London Asians. Thorax 1995;50:175-80.

(44) Crowle AJ, Ross EJ, May MH. Inhibition by 1,25(OH)2-vitamin D3 of the

multiplication of virulent tubercle bacilli cultured in human macrophages. Infect Immunity 1987;55:2945-50.

(45) Hnizdo E, Murray J. Risk of pulmonary tuberculosis relative to silicosis and exposure to silica dust in South African gold miners (vol 55, pg 496, 1998). Occupational and Environmental Medicine 1999; 56(3):215-216.

(46) Opsahl R, WESSELAA.T, RIDDERVO.HO. Pulmonary Tuberculosis in Mitral Stenosis and Diabetes Mellitus. Acta Tuberculosea et Pneumologica Scandinavica 1961; 40(4):290-&.

(47) Small PM, Shafer RW, Hopewell PC, Singh SP, Murphy MJ, Desmond E et al. Exogenous Reinfection with Multidrug-Resistant Mycobacterium-Tuberculosis in Patients with Advanced Hiv-Infection. New England Journal of Medicine 1993; 328(16):1137-1144.

(48) Cantwell MF, McKenna M, McCray E, Onorato IM. Tuberculosis and race/ethnicity in the United States - Impact of socioeconomic status. American Journal of Respiratory and Critical Care Medicine 1998; 157(4):1016-1020.

(49) Combating TB - The DOTS strategy. Available at URL: http://w3.whosea.org/tb/dots.htm

(50) Dye C, Garnett GP, Sleeman K, Williams BG. Prospects for worldwide tuberculosis control under the WHO DOTS strategy. Directly observed short-course therapy. Lancet 1998; 352(9144):1886-1891.

(51) Issues relating to the use of BCG in immunization programmes. Chapter 7. BCG efficacy. Available at URL: http://www.who.int/vaccine_research/documents/en/bcg_vaccines.pdf

(52) Pheiffer C, Betts JC, Flynn HR, Lukey PT, van Helden P. Protein expression by a Beijing strain differs from that of another clinical isolate and Mycobacterium tuberculosis H37Rv. Microbiology-Sgm 2005; 151:1139-1150.

(53) Glynn JR, Whiteley J, Bifani PJ, Kremer K, van Soolingen D. Worldwide occurrence of Beijing/W strains of Mycobacterium tuberculosis: A systematic review. Emerging Infectious Diseases 2002; 8(8):843-849.

(54) Mokrousov I, Narvskaya O, Otten T, Vyazovaya A, Limeschenko E, Steklova L et al. Phylogenetic reconstruction within Mycobacterium tuberculosis Beijing genotype in northwestern Russia. Research in Microbiology 2002; 153(10):629-637.

(55) vanSoolingen D, Qian LS, Dehaas PEW, Douglas JT, Traore H, Portaels F et al. Predominance of A Single Genotype of Mycobacterium-Tuberculosis in Countries of East-Asia. Journal of Clinical Microbiology 1995; 33(12):3234-3238.

(56) Dahle UR, Sandven P, Heldal E, Mannsaaker T, Caugant DA. Deciphering an outbreak of drug-resistant Mycobacterium tuberculosis. Journal of Clinical Microbiology 2003; 41(1):67-72.

(57) Anh DD, Borgdorff MW, Van LN, Lan NT, van Gorkom T, Kremer K et al. Mycobacterium tuberculosis Beijing genotype emerging in Vietnam. Emerg Infect Dis 2000; 6(3):302-305.

(58) van Soolingen D, Kremer K, Borgdorff M. Mycobacterium tuberculosis Beijing genotype, Thailand--reply to Dr. Prodinger. Emerg Infect Dis 2001; 7(4):763-764.

(59) Kremer K, Glynn JR, Lillebaek T, Niemann S, Kurepina NE, Kreiswirth BN et al. Definition of the Beijing/W lineage of Mycobacterium tuberculosis on the basis of genetic markers. Journal of Clinical Microbiology 2004; 42(9):4040-4049.

(60) Kamerbeek J, Schouls L, Kolk A, vanAgterveld M, vanSoolingen D, Kuijper S et al. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. Journal of Clinical Microbiology 1997; 35(4):907-914.

(61) Kremer K, Glynn JR, Lillebaek T, Niemann S, Kurepina NE, Kreiswirth BN et al. Definition of the Beijing/W lineage of Mycobacterium tuberculosis on the basis of genetic markers. J Clin Microbiol 2004; 42(9):4040-4049.

(62) Vietnam information. Available at URL: http://www.geographycards.com/countryinfo/vm.html#Ge

(63) WHO/UNICEF. WHO/UNICEF Review of National Immunization Coverage. 8-25-2005.

(64) Issues relating to the use of BCG in immunization programmes. Available at URL: http://www.who.int/vaccine_research/documents/en/bcg_vaccines.pdf

(65) WHO. Global Tuberculosis Control. Who report 2005. p141-144. Available at URL: http://www.stoptb.org/countries/GlobalReport2005/vietnam.pdf

(66) Kenneth J.Rothman. Epidemiology an introduction. Oxford unversity press Inc., 198 Madison Avenue, New York, New York 10016, 2002.

(67) Charles H.hennekens MDDrPHJEBScDEbSLMPhD. Epidemiology in Medicine. 2006.

(68) Centre for Evidence-Based Medicine IoHSUoO. Study design. 2006.

(69) Charles H.hennekens MDDrPHJEBScDEbSLMPhD. Design Strategies in Epidemiologic Research. 1987.

(70) WHO/UNICEF Review of National Immunization Coverage - 25 August, 2005.: 2007.

(71) Meenu Singh NVAMLKaMS. Role of Gastric Lavage and Broncho - Alveolar Lavage in the Bacteriological Diagnosis of Childhood Pulmonary Tubeculosis. 2006. Indian Pediatrics - Editorial.

(72) National Institute of Hygiene and Epidemiology - Holand medical department. Training document: Research methodology and science report writing. Vietnam community health training and consulting network, 2006. 2006.

(73) The citation for the mathematical formula (sample size): INNFØRING I STATISTIKK med medisinske eksempler by Odd O. Aalen, Ad Notam Gyldendal AS, 2. edition, 1994. 1994.

(74) Pomputius WF, Rost J, Dennehy PH, Carter EJ. Standardization of gastric aspirate technique improves yield in the diagnosis of tuberculosis in children. Pediatric Infectious Disease Journal 1997; 16(2):222-226.

(75) Detection of Mycobacterium tuberculosis in Gastric Aspirates Collected From Children: Hospitalization Is Not Necessary . Available at URL: http://pediatrics.aappublications.org/cgi/content/full/102/4/e40

(76) IUATLD/WHO guidelines. AFB MICROSCOPY METHOD IN DOTS. DIAGNOSTIC LABORATORY METHODS FOR TUBERCULOSIS. 2007.

(77) World Health Organization Geneva. Guideline for surveillance of drug resistance in tuberculosis. 2003.

(78) Kamerbeek J, Schouls L, Kolk A, vanAgterveld M, vanSoolingen D, Kuijper S et al. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. Journal of Clinical Microbiology 1997; 35(4):907-914.

(79) Kristin Kremer. "Spoligotyping" aPCR-based method to simultaneously detect and typecomplex bacteria. 2002.

(80) van Soolingen D. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. Journal of Internal Medicine 2001; 249(1):1-26.

(81) Dahle UR, Sandven P, Heldal E, Caugant DA. Molecular epidemiology of Mycobacterium tuberculosis in Norway. Journal of Clinical Microbiology 2001; 39(5):1802-1807.

(82) Dick van Soolingen, Petra E.W.de Haas, Kristin Kremer. Restriction fragment length polymorphism (RFLP) typing of mycrobacteria. 2007.

(83) Mark Woodward. Epidemiology study design and data analysis, second edition, Basic analytical procedure 103-106. Chapmann & Hall/CRC, 2007.

(84) *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. additional file. BMC Microbiol 2006; 6: 23 2007.

(85) Lawn SD, Afful B, Acheampong JW. Pulmonary tuberculosis: diagnostic delay in Ghanaian adults. International Journal of Tuberculosis and Lung Disease 1998; 2(8):635-640.

(86) Johansson E, Long NH, Diwan VK, Winkvist A. Gender and tuberculosis control Perspectives on health seeking behaviour among men and women in Vietnam. Health Policy 2000; 52(1):33-51.

(87) WHO. WHO report 2006. 2007.

(88) Hudelson P. Gender differentials in tuberculosis: The role of socio-economic and cultural factors. Tubercle and Lung Disease 1996; 77(5):391-400.

(89) Long NH, Johansson E, Lonnroth K, Eriksson B, Winkvist A, Diwan VK. Longer

delays in tuberculosis diagnosis among women in Vietnam. International Journal of Tuberculosis and Lung Disease 1999; 3(5):388-393.

(90) Lonnroth K, Thuong LM, Linh PD, Diwan VK. Delay and discontinuity - a survey of TB patients' search of a diagnosis in a diversified health care system. International Journal of Tuberculosis and Lung Disease 1999; 3(11):992-1000.

(91) Wandwalo ER, Morkve O. Delay in tuberculosis case-finding and treatment in Mwanza, Tanzania. International Journal of Tuberculosis and Lung Disease 2000; 4(2):133-138.

(92) Homolka J, Krejbich F. Results of tuberculin skin test in 1,172 cases of bacteriologically confirmed tuberculosis. Chest 2006; 130(4):275S.

(93) Hennessey KA, Schulte JM, Cook L, Collins M, Onorato IM, Valway SE. Tuberculin skin test screening practices among US colleges and universities. Jama-Journal of the American Medical Association 1998; 280(23):2008-2012.

(94) Demissie M, Lindtjorn B, Berhane Y. Patient and health service delay in the diagnosis of pulmonary tuberculosis in Ethiopia. Bmc Public Health 2002; 2.

(95) Epidemiologic Basis of Tuberculosis Control. IUATLD 2007.

(96) **Diagnostic laboratory methods for tubeculosis**. 2007.

(97) Foulds J, O'Brien R. New tools for the diagnosis of tuberculosis: the perspective of developing countries. International Journal of Tuberculosis and Lung Disease 1998; 2(10):778-783.

(98) Huong NT, Lan NTN, Cobelens FGJ, Duong BD, Co NV, Bosman MC et al. Antituberculosis drug resistance in the south of Vietnam: Prevalence and trends. Journal of Infectious Diseases 2006; 194(9):1226-1232.

(99) Kristin Kremer., Annelies Bunschoten, Leo Schouls, Dick van Soolingen, Jan van Embden. "SPOLIGOTYPING" a PCCR - based method to simultaneously detect and typr Mycobacterium tuberculosis complex bacteria. Research laboratory for infectious diseases, National institute of Public health and the Environment, P.O. Box 1, 3720 BA, Bilthoven, The Netherlands, 2007.

ANNEX 1: MOLECULAR METHODS

Laboratory method:

Molecular biology analysis will be carried out at NIPH, Norway in the 1st quarter of 2007 because of PCR, Spoligotyping and RFLP are not routine analysis in home country during the research time.

1 Isolation of high molecular weight genomic DNA from *Mycobacteria*

The method of choice is determined by the amount of starting material, the desired amount and purity of DNA isolated, and the nature of the materials from which the DNA is to be extracted. DNA isolated by most miniprep protocols contains significant RNA contamination which can interfere with DNA digestion and with hybridization of the southern blots. RNA can be removed either by digestion of the entire sample with DNase-free RNase after preparation, or at the same time as restriction endonuclease digestion (20).

Isolation and extraction of high molecular weight genomic DNA from Mycobacterium (82).

Caution: for mycobacterium pathogenic to humans appropriate containment facilities should be used while handling before heat-inactivation.

Transfer at least two loops of mycobacterium into a micro centrifuge tube containing 400 µl of 1X TE
Kill the cells by heating for 20 minutes at 80°C, and cool at RT
Put the CTAB/NaCl solution at 65°C, (for use in step 6)
Add 5 µl proteinase K (10 mg/ml) – mix lightly
Add 70 µl of 10% SDS, mix them and vortex, and incubate for 10 – 15 minutes at 65°C
Add 100 µl of 5M NaCl and 100 µl of pre-warmed CTAB/NaCl solution (step 3), and vortex until the liquid content becomes white ("milky") (or mix them 10 – 20 seconds).
Incubate for 10 minutes at 65°C
Add 750 µl of Chloroform/isoamyl alcohol and vortex for at least 10 seconds
Centrifuge for 5 minutes at 13,000 rpm
Transfer the aqueous phase (upper layer) to a fresh micro centrifuge tube, by pipetting small aliquots of e.g. 180 µl
Add 450 µl (0.6 volume) of isopropanol – mix well
Manually move the tube slowly upside down to precipitate the nucleic acids and estimate the amount of 1X TE in which the DNA should be redissolved in step 20. write the estimated volume e.g. on the tube
Place at -20°C for at least 30 minutes (± over night)

Extraction

Take out from Freezer, place it in RT

Centrifuge in 10 minutes at 13,000 rpm
Discard most of supernatant, leave about 20 µl (3mm height) above the pellet
Add 900 µl of cold 70% Ethanol (from the -20°C freezer) and turn the tube a few time upside down to wash the DNA precipate. Note: 70% (70ml 100% and 30 ml H2O)
Centrifuge in 10 minutes at 13,000 rpm
Discard most of the supernatant; leave about 20 µl (3 mm height) above the pellet
Upside down the tube and dry at 37°C in 30 minutes

Add 20 µl of TE buffer (1X TE) to dissolve DNA
Vortex
Place in freezer – 20^{o}C

2. DNA concentration determination

Before further analysis, it is important to determine the concentration and condition of the DNA that you have isolated. This can be done in three ways: by examining UV absorbance with spectrophotometer, by fluorimetry, or by comparison with DNA standard on agarose gels.

DNA prepared by miniprep protocols often contains a large amount of RNA and pigments that can cause spuriously high estimation of DNA concentration on a spectrophotometer. For this reason 0.8% agarose gels are useful for assessing both the quantity and the quality of genomic DNA (is it high molecular weight, or is these substantial shearing or degradation?) and the amount of RNA present (20).

DNA check

Prepare 0.8% gel - 1.2 gram ME Agarose
150 ml TBE 1X buffer
boiling 2 a.m chemicals together in micro oven
and get cold at 60^{o}C
Solidify in 30 – 45 minutes
100 V = program 1

Make a list of samples
Take samples out of Freeze
Labeling new tubes with as same as samples
Melt samples, then take out of each sample 1 µl add to new tube
Add 9 µl 1X DNA sample buffer (^{m}/Rnaze)
Apply 10 µl of gel (+ 5µl ladder)
Run program 1 (2 hours) Can keep overnight at 4^{o}C
Dye Gel with ^{m}/ ethidium bromid in 15 minutes
Dye off (shake) with distilled water in 20 minutes (or 10 minutes + 10 minutes)
Photographer (82)

3 Restriction endonuclease digestion of DNA

Digestion of DNA with restriction endonuclease that cleave at different recognition sites lies at the heart of RFLP analysis. Complete digestion is essential to obtaining interpretable RFLP patterns. Several factors affect endonuclease activity, including pH, concentration, and type of ions in the buffer, and the temperature of the reaction (20).

Cutting DNA with restriction enzyme

We use PVU II (Restriction endonuclease) in order to cut DNA as follow:

CAG|CTG

GTC|GAC

Take out samples from freeze

Labeling new eppendofts

Transfer 9 µl DNA to each eppendoft and place on the ice
Add 2 µl M buffer
Add 8 µl distilled water
Add 1 µl PVU II, mixes

Mixes

Centrifuge at 1300 rpm (increase to 1300 rpm and slow down)

Put all these eppendoft to water-bath at 37^0C in 2 hours or over night

Note: If the DNA concentration weak, we can increase the volume of DNA more than 9 µl and decreased the volume of distilled water – but the total of DNA and distilled water must be 17 µl

Add 5 µl 1x DNA sample buffer ^{m}/ RNA'se

Put into refrigerator (2-8^0C)

Could be kept in several months, but avoid to dry samples (82)

4 Separate DNA fragment with electroforese "night gel"

The size range of fragments that results from digestion of most DNAs makes agarose the idea matrix for size fractionation of the resulting DNA fragments by electrophoresis. Gels of 0.8% agarose are useful for the broadest range of fragment size encountered in routine analysis (20).

Make 0.8% gel:

1.2 gram ME Agarose
150 ml TBE 1X buffer
Use distilled water (pick up at 1st floor) whenever keep gel and buffer bottle
Add 8 µl ladder 1:5
Add 12 µl of sample
Run program 2 (100v in 10 minutes, 25v in 18 hours)
Dye gel and photograph
We could use vacuum blotting (82).

DNA transfer to membrane

DNA fragments are generally transferred from the electrophoresis gel matrix and bound to a membrane for subsequent hybridization to the probe of choice. For all protocols, the quality of the resulting membrane is dependent upon careful handling of the gel, membrane, and filter paper that comes in contact with the membrane (20).

Vacuum Blotting

Principle:

Vacuum gene XL is a system in order to transfer acid nucleic from gel to membrane (gene screen).

Rapid carry out:

Method namely Southern Blotting, it combines electroforese gene (analysis DNA fragments by size) and hybridization with sample, it find out DNA sequence

First of all, DNA is disengaged to simple DNA fibers and put into nylon membrane. After blotting, all fibers of DNA of gene will be transferred and fixed in membrane.

UV fixed DNA to membrane (DNA face down)

Use Gloves

Cut gene screen 11 x 17 cm

Damp screen (a soft polyethylene) by water, put it into o square pot, smooth face on top

Put the membrane into middle of screen

Mask of 0.15 mm polyethylene with a window appropriate with membrane

Cut Gel and put over the mask

Put square pot to the mould

Close 4 clips

Fit a plastic pipe in to mould, make sure it is close tight

Tight of glass bottle and start vacuum

Keep the pressure about 50 mbar

Flush 50 ml 0.25 M HCL (cool HCL) on the Gel, make sure that the depurination solution cover all gel

Keep in 7 minutes

Lean the square pot, use the glove to push out the HCL from gel

Suck out HCL

Close the pipe

Keep the pressure at 50 mbar again

Flush 50 ml 0.5 MNAOH ^{m}/1.5 MNACL (denaturalization solution) in to the Gel

And make sure the solution cover all gel

Keep 20 minutes

Check the pressure of vacuum at 50 mbar

Lean the square pot, push out the solution by finger

Suck out solution

Close the pipe

Keep the pressure at 50 mbar again

Flush 50 ml 1M tris HCL ^{m}/1.5 MNACL pH 7.5 (neutralization solution) cover all gel

Keep in 20 minutes, make sure the pressure at 50 mbar

Suck out the solution

Flush 750 ml 20X SSC (transfer solution) until cover black mark of mould, avoid the gel floating

Keep 60 minutes

Make sure the vacuum pressure at 50 mbar

Suck out the solution

Put out the gel, turn off the vacuum

Put the gene screen on the filter paper, dry it in RT about 30 minutes (may change the filter paper at this time)

Put the gene screen on the dry and clean filter paper and pack it with plastic paper

UV illuminuteate in 5 minutes (DNA face down without plastic)

Keep in refrigerator

Note: all bottles needs to be kept in refrigerator (82).

6 Hybridisation with Probe

This plastic membrane will be hybridized in a solution which is included a probe namely dig and a ladder namely dig.

The probe must be included the DNA sequence which are we want to find out. When we increase the temperature of probe? Sample will be denatured and hybridized with the same basic XL. The materials of probe/ samples which are not combined together will be losing after flush….

Solution for pre-hybridization

Use for 1 filter

- 1g blocking reagent (in refrigerator)
- 25 ml 20 X SSC (RT)
- 1 ml 10% Sarcosyl (in refrigerator)
- 200 µl 10% SDS (RT)
- Full fill 1000 ml ^{m}/sterile water (before SDS)

Mix them in an infusion bottle of 500 ml distilled water

Put and shake in the water bath 68^0C in one hour

Take out the pre-hybridization from water bath…

Put the filter to a plastic box (make sure the filter fit with plastic box and DNA face down)

Add 80 ml pre-hybridization to plastic box and keep the rest of liquid

Put the lid on the plastic box

Place and shake the plastic box in water bath 60^0C in 2 hours

Note: put a heavy material over the plastic box

Hybridization solution

25 µl dig marked probe (keep in freeze – 20^0C)

2,5 µl dig marked ladder (keep in freeze – 20^0C)

10 ml pre-hybridization

Dig marked probe and ladder keep in freeze of laboratory, make warm and place on the ice before use

After use with pre-hybridization, can freeze and reuse for 3 times. Make sure to mark number of use

Take out the hybridization from freeze, make warm at RT or refreeze

Denaturisation probe and ladder before hybridization

Add warmed solution to glass tube with lid

Boiling in microwave oven, make sure fill solution as least a haft of glass tube

Put into the ice in 10 minutes

(note: make sure enough 10ml hybridization solution)

Use a big glass tube with green lid for hybridization (gloves)

Take out the filter box form water bath

Role up the filter as same as cigar

Use a pinsett put the cigar filter to glass tube

Use glass pipette take out the air bubbles if needed

Add denaturisation to the filter glass tube

Put the glass tube to the hybridization machine, at 60^0C overnight

(note: we need to put a other glass tube with the same volume at opposite side)

Clean plastic boxes with soap and dry

Next day:

Turn off the hybridization machine; take out the Filter glass tube

Clean the filter and make colors in order to see DNA

Clean all tube with soap, and dry

7 Buffers will be used for cleaning and dye colors

Sterile bottle and tube must be placed at glass cleaner room some days before

Put in to the water bath with buffer 2 in 1 hour at 68^0C

Marked one bottle (1L): *0.1x SSC / 0.1 % SDS (measure level)*

About 5 ml 20 X SSC
10 ml 10% SDS (last one)
Full fill 1L ^{m}/ distilled water
Mix and lid sterile

Marked one bottle (1L): *2x SSC / 0.1 % SDS (measure level)*

100 mg 20 X SSC
10 ml 10% SDS (last one)
Full fill 1L ^{m}/ distilled water
Mix

Marked one bottle (1L): ***Buffer 1*** and measure level
100 ml tri HCL pH 7.5 (refrigerator)
50 ml 3M NACL (refrigerator)
Full fill 1L ^{m}/ distilled water
Mix

Only use in one day (all 3 above mention bottles)/ except SSC in RT

Marked one bottle (1L): Buffer 2 (measure level)
1g of blocking reagent (refrigerator)
200 ml buffer 1
Dissolve in water bath 68^{0}C in 1 hour

Marked one bottle Buffer 3 (measure level)
100 ml 1 M Tri Hcl pH 9.5 (refrigerator)
33.3 ml 3M Nacl (refrigerator)
50 ml 1 M Mgcl2 (refrigerator)
Full fill 1L ^{m}/ distilled water
(Can be used in 1 month – marked the date on the bottle – keep in RT)

Turn on the water bath with shake at 68^{0}C

Use gloves
Take out the hybridization tube from hybridization machine
Add to plastic box 100 ml 2 X SSC / 0.1 % SDS
Use pinsett put out the filter from glass tube and place to the above mention box (the rest of hybridization liquid can be keep in other tube and frozen)
Shake the tube and through out the liquid
Add 500 ml 2X SSC / 0.1 % SDS
Shake 5 minutes at RT, 2 times (make sure the speed of shaking machine, avoid through out liquid)
Through out liquid after shaking, repeat 1 time
Add 500 ml 0.1 X SSC / 0.1 % SDS
Shake twice in the water bath in 20 minutes at 68^{0}C, through out the liquid
Now, use o big plastic cover Petri, place the filter inside
Add 100 ml Buffer 1
Shake in 1 minute at RT
Through out buffer 1
Add 200 ml buffer 2
Shake in 30 minutes at RT
Through out buffer 2
Add 100 ml Buffer 1
Shake 1 minute at RT
Through out buffer 1
Add exactly 80 ml buffer 1 and {16 µl DIG DNA labeling and detection kit} direct to the

plastic cover Petri on the shaking machine ($N^0 8$)
Shake 30 minutes at RT
Through out liquid
Rinse twice 15 minutes in 100 ml buffer 1 (shake)
Rinse 2 minutes with 80 ml buffer 3

Prepare dye solution exactly:

40 ml buffer 3
Add 800 µl NBT / BCIP (freeze – $N^0 9$)

Put in to one box and put the filer in (place the DNA side down)
Pack the filter box in aluminuteum paper, place in dark (drawer) in 24 hours
Rinse filter with distilled water

8 Prepare the STOP – MIX solution

3 ml tris/Hcl – buffer 1.0M pH 8.0
1.5 ml 0.2M EDTA NA_2 $2H_20$
295 ml distilled water
Measure water level in 500 ml tube

Through out dye solution
Rinse sometime with distilled water
Rinse sometime with STOP – MIX solution
Fill in the rest of solution in box, keep filter in the box some hours (can be left for several days)
Place filter on the filter paper at RT to dry
Clean all tube with water (82).

II Spoligotyping:

The typing method is based on DNA polymorphism present at one particular chromosomal locus, the "Direct Repeat" (DR) region, which is uniquely present in *mycobacterium tuberculosis complex* bacteria. With the method described here, the presence or absence in the DR of 43 spacer of known sequence can be detected by hybridization of PCR-amplified spacer DNA to a set of immobilized ologonucleotides, representing each of the unique spacer DNA sequences. This method will be referred to as *spoligotyping* (from *spacer oligotyping*)

1 PCR: Polymerase chain reaction (PCR) – Gene amplification methods (PCR as well as isothermal) developed for the diagnosis of tuberculosis is highly sensitive especially in culture-negative specimens from *paucibacillary* forms of disease. A variety of PCR techniques have been developed for detection of specific sequences of *Mycobacterium*

tuberculosis and other *Mycobacteria* (19). PCR defined segments of DNA can be amplified to microgram quantities from as little as single template molecule. Although the procedure is in some way deceptively simple, and the reaction can entail complex biochemical interactions, it is in most application a fast, relatively inexpensive and easy way to generate ample materials for further analysis (20).

In this research, the last region of the Mtb genome, where is located the direct repeats namely A; B; C; D will be amplified by primes DRa and prime DRb. Amplification of the spacers is accomplished by using the primers DRa and DRb, which enable to amplify the whole DR (direct repeat) region. Only a very small amount of template DNA is required. Typically the PCR is performed on 10ng purified chromosomal Mycobacterial DNA but, with minor adaptations, DNA extracts from clinical samples from freezer can server as template. The PCR products are labelled with Biotin, because one of the primers is biotinylated. The primers of PCR are based on the DR sequence:

DRa: 5' – GGT TTT GGG TCT GAC GAC – 3', biotinylated at 5' end

DRb: 5' – CCG AGA GGG GAC GGA AAC – 3'

Procedure:

Dilute the DNA samples to the required concentration. Always include chromosomal DNA of M.tuberculosis strain H37Rv and M.bovis BCG as positive controls. Use water as a negative control (99).

Prepare the reaction mixture:			Place the tubes in a PCR-apparatus for amplification, and perform the following temperature cycling:
DNA product	02	µl	
10x PCR	05	µl	
Primer DRa	04	µl	2 minutes at 96^0C
Primer DRb	04	µl	1 minute 96^0C
dNTP mix	04	µl	1 minute 55^0C
Tag	0.25	µl	30 seconds 72^0C } 30 cycles
H_20	30.75	µl	5 minutes at 72^0C

2 Hybridization with PCR product and detection

Hybridization of biotin-labeled PCR products to the immobilized spacer-oligos that represent spacers of known sequence. The presence pf spacers are visualized on film as black squares after incubation with streptavidin-peroxidase and ECL-detection.

Procedure:

All buffers should be pre-warmed before use. Prepare the following buffer from concentrated stocks, using demineralised water for dilution (quantities for one membrance):

250 ml 2xSSPE/0.1% SDS, 60^0C

500 ml 2xSSPE/0.5% SDS, 60^0C

500 ml 2xSSPE/0.5% SDS, 42^0C

500 ml 2xSSPE, room temperature

Add 25 µl of PCR products to 150 µl 2xSSPE/0.1% SDS

Heat-denature the diluted PCR products for 10 minutes at 99^0C and cool on ice immediately

Wash the membrane for 5 minutes at 60^0C in 250 ml 2xSSPE/0.1% SDS

Place the membrane and a support cushion into the miniblotter, in such a way that the slots are perpendicular to the line pattern of the applied oligonucleotides.

Remove residual fluid from the slots of the miniblotter by aspiration

Fill the slots with diluted PCR product (avoid air bubbles) and hybridize for 60 minutes at 60^0C on a horizontal surface (no shaking). Avoid contamination of neighbouring slots.

Remove the samples from the miniblotter by aspiration and take the membrane from the miniblotter using forceps

Wash the membrane twice in 250ml 2xSSPE/0.5% SDS for 10 minutes at 60^0C

Place the membrane in a rolling bottle and allow it to cool down to prevent inactivation of the peroxidase in the next step

Add 2.5 µl streptavidin-peroxidase conjugate (500U/ml) to 10 minutes at 42^0C

Rinse the membrane twice with 250 ml of 2xSSPE for 5 minutes at room temperature

For chelmiluminiscent detection of hybridizing DNA, incubate the membrane for 1 minute in 20 ml ECL detection liquid (10ml ECL 1 and 10 ml ECL 2)

Cover the membrance with a transparent plastic sheet or saran-wrap and expose a light sensitive film to the membrane for 20 minutes.

If the signal too weak or too strong the membrane can be used again directly to expose another film for a shorter or longer period (99).

ANNEX 2

DATA COLLECTION FORM

University of Oslo, Norway | **National Institute of hygiene & Epidemiology** | **National Tuberculosis & Lung Diseases**

DATA COLLECTION FORM

Date of admission: ...../...../..... *Study code:* .....

Department:..... Archive No:.....

Bed No:..... Medical code...../...../.....

General Information

1 Full name: ..... 2 Date of birth …/…/……

3 Gender (1) Male: ❑ (2)Female: ❑ 4 Ethnic: .....

5 Address: No.....Street.....Commune.....

District.....City.....

6 Nationality (1) Vietnam ❑ (2) Foreigner ❑

7 Place of work .....

8 Telephone No (1) Desk..... (2) Mobile.....

9 Smoker (1) No ❑ (2) Yes ❑

10 Alcohol (1) No ❑ (2) Yes ❑

11 TB related to contact persons: (1) No ❑ (2) Yes ❑

12 History of Immunization of BCG: (1) with scar ❑ (2) No scar ❑

(3) Immunization card ❑ (4) Other ❑ .....

Symptoms

13		a	b	c	d	e	f	g
		No	Yes	> 1 week	1-4 week	1-6 m	1/2 -1 year	> 1 year
a	Spitting & Sputum	❑	❑	❑	❑	❑	❑	❑

b	Cough	❑	❑	❑	❑	❑	❑	❑
c	Fever	❑	❑	❑	❑	❑	❑	❑
d	Chest pain	❑	❑	❑	❑	❑	❑	❑
e	Fatigue	❑	❑	❑	❑	❑	❑	❑
f	Weight loss	❑	❑	❑	❑	❑	❑	❑

14 Other clinical symptoms if any

History

15 Have you been treated for TB .....time before? When, Where, for how long, Result?

Paraclinic information

16	Sputum examination	Result 1	(1) Positive	❑	(2) Negative	❑
		Result 2	(3) Positive	❑	(4) Negative	❑
		Result 3	(5) Positive	❑	(6) Negative	❑
17	Culture	Result	(1) Positive	❑	(2) Negative	❑
18	Mantoux	Result	(1) Positive	❑	(2) Negative	❑
19	HIV	Result	(1) Positive	❑	(2) Negative	❑

20 X-ray

CT

21 Scanner:

22 Ultrasound

23 PCR:

24 RFLP

	(1) No	(2) Yes
Clustered strains:	❑	❑

No of copy IS6110:

Patient's signature Name of patient

Signature of interviewer Name of patient

Signature of study Director Name of patient

University of Oslo, Norway | **National Institute of hygiene & Epidemiology** | **National Tuberculosis & Lung Diseases**

DATA COLLECTION FORM (SOURCE OF INFECTION)

Study code……..

Department:..... Archive No …….

Bed No Medical code……/……/

Full name: ..... 2 Date of birth …./…./……

Source of TB

25 ***No of TB infected in the family*** ……

26 Full name: ..... 27 Date of birth …./…./……

(1) Male:

28 Gender ❑ (2) Female: ❑ 29 Ethnic: .....

30 Address: No.....Street.....Commune.....

District.....City.....

(2)

31 Nationality (1) Vietnam: ❑ Foreigner ❑

32 Place of work .....

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33 Full name: ..... 34 Date of birth …./…./……

(1) Mal

35 Gender e: ❑ (2) Female: ❑ 36 Ethnic: .....

37 Address: No.....Street.....Commune.....

District.....City.....

(2)

38 Nationality (1) Vietnam: ❑ Foreigner ❑

.....

39 Place of work .....

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40 ***No of people being infected with TB*** ……

41 Full name: ..... 42 Date of birth …./…./……

(1) Mal

43 Gender e: ❑ (2) Female: ❑ 44 Ethnic: .....

45 Address: No.....Street.....Commune.....

District.....City.....

46 Nationality (1) Vietnam: ❑ (2) ❑

Foreigner

47 Place of work .....

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48 Full name: ..... 49 Date of birth …./…./……

(1) Mal

50 Gender e: ❑ (2) Female: ❑ 51 Ethnic: .....

52 Address: No.....Street.....Commune.....

District.....City.....

53 Nationality (1) Vietnam: ❑ (2) Foreigner ❑

54 Place of work .....

ANNEX 3

CONSENT FORM

I am …….. from a research team established by Department of International Health, University of Oslo-Norway, NTRH and Molecular Biology Department - NIHE. I am here to conduct a study on Tuberculosis among children less than 15 years and adult over 40 years of age admitted to National Institute of Tuberculosis from September to November 2006. The study is trying to find out the different successful strains of Mycobacterium Tuberculosis between children and adult. If BCG proves inactive in preventing new strains of MTB, a new or modified vaccine will be needed for EPI program in Vietnam in the future. I would like to interview you, and ask you for your permission to collect MTB strains sample (Gastric lavage) from you and your own child.

I have a few questions about Tuberculosis and related issues. Your answers will be written and then used for analysis. All information you provide will be handled as confidential and your personal answers will not be known, except by the interviewer and the coordinator of this study. The results will be used only to improve strategies for prevention of Tuberculosis by vaccination, one of the most burden diseases in Vietnam.

We will need at least 30 minutes to discuss and record the information. You can withdraw from the interview at any stage without any consequence if you do not wish to continue.

Will you participate in this study? Yes □ No □

Do you have any question?

Thank you.

Date: …../……./2006.

Interviewee's signature: …… **Interviewee's signature**: ……

Interviewee's signature: …… **Interviewee's signature**: ……